Molecular and Biochemical Diagnosis of Cerebral Creatine Deficiency Syndromes

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Molecular and Biochemical Diagnosis of Cerebral Creatine Deficiency Syndromes

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geboren te Porto, Portugal

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Eles não sabem que o sonho
é uma constante da vida
tão concreta e definida
como outra coisa qualquer,
como esta pedra cinzenta
em que me sento e descanso,
como este ribeiro manso
em serenos sobressaltos,
como estes pinheiros altos
que em verde e oiro se agitam,
como estas aves que gritam
em bebedeiras de azul.

Eles não sabem que o sonho
é vinho, é espuma, é fermento,
bichinho álacre e sedento,
de focinho pontiagudo,
que fossa através de tudo
num perpétuo movimento.

Eles não sabem que o sonho
é tela, é cor, é pincel,
base, fuste, capitel,
arco em ogiva, vitral,
pináculo de catedral,
contraponto, sinfonia,
máscara grega, magia,
que é retorta de alquimista,
mapa do mundo distante,
rosa-dos-ventos, Infante,
caravela quinhentista,
que é cabo da Boa Esperança,
ouro, canela, marfim,
florete de espadachim,
bastidor, passo de dança,
Colombina e Arlequim,
passarola voadora,
pára-raios, locomotiva,
barco de proa festiva,
alto-forno, geradora,
cisão do átomo, radar,
ultra-som, televisão,
desembarque em foguetão
na superfície lunar.

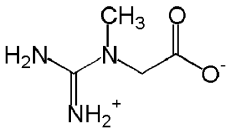
Eles não sabem, nem sonham,
que o sonho comanda a vida,
que sempre que um homem sonha
o mundo pula e avança
como bola colorida
entre as mãos de uma criança

Pedra Filosofal *in* Movimento Perpétuo

CONTENTS

ABBREVIATIONS

AGAT	arginine:glycine amidinotransferase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
CCDS	cerebral creatine deficiency syndromes
Cr	creatine
Crn	creatinine
Cr/Crn	creatine/creatinine ratio
CK	creatine kinase
GAA	guanidinoacetic acid
GAMT	guanidinoacetate methyltransferase
HPLC	high performance liquid chromatography
MR	mental retardation
OMIM	Online Mendelian Inheritance in Man
ORF	open reading frame
PCr	phosphocreatine
proton MRS	proton magnetic resonance spectroscopy
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SID GC-MS	stable isotope dilution gas chromatography-mass spectrometry
SLC6A8	solute carrier family 6 member 8 (creatine transporter)
XLMR	X linked mental retardation



Creatine

Chapter 1

INTRODUCTION

Creatine

The compound α-methylguanidino acetic acid was first described in the year 1835 and was named creatine (Figure 1). Creatine biosynthesis and metabolism have been studied for about 100 years and their importance has been recognized since then.

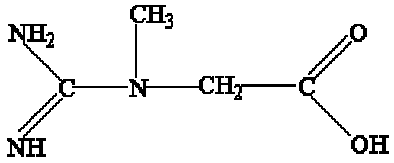


Figure 1. Chemical structure of creatine.

Creatine is distributed throughout the body: 95% of the creatine pool is found in skeletal muscle, while the remaining 5% is found in brain, liver, kidney and testis [1]. Creatine body content is maintained by both nutritional intake (50%) and biosynthesis (50%).

Creatine biosynthesis involves a two-step reaction. The first, which is rate limiting, is catalyzed by arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1) and involves the transfer of the amidino group of arginine to glycine, yielding ornithine and guanidinoacetic acid. In the second step, guanidinoacetic acid is methylated into creatine by guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2) [1,2] (Figure 2). Creatine is distributed via the blood stream and taken up in the different tissues by the creatine transporter (SLC6A8) (Figure 2, insert). Creatine kinase catalyses the phosphorylation and dephosphorylation of creatine and phosphocreatine, respectively, providing a high energy phosphate buffering system during adenosine triphosphate release and use [3]. Intracellular creatine and phosphocreatine are non-enzymatically converted to creatinine with a constant daily turnover of 1.5% of the creatine pool. The reversible and nonenzymatic cyclization of creatine to creatinine is pH and temperature dependent. Creatinine is excreted in urine and its daily excretion is directly proportional to the total body creatine pool.

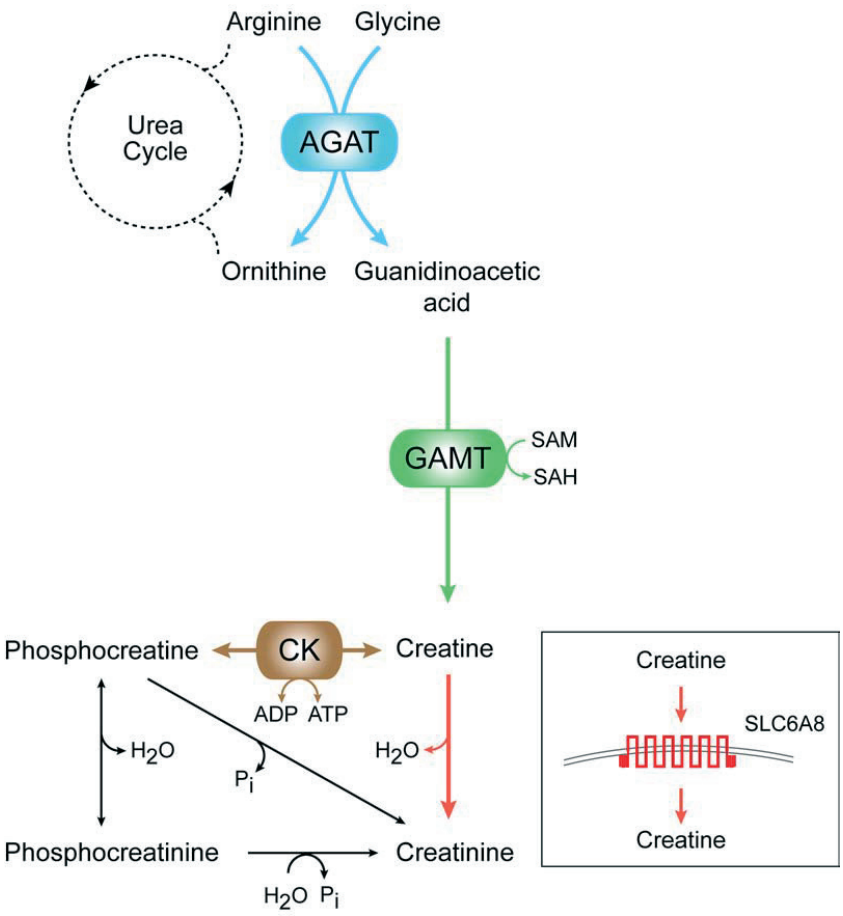


Figure 2. Creatine metabolic pathway.

Creatine metabolism and disease

Primary creatine metabolism disorders- Cerebral Creatine Deficiency Syndromes

Cerebral Creatine Deficiency Syndromes (CCDS) are a group of disorders consisting of defects in proteins involved in creatine biosynthesis, AGAT and GAMT, and in its transporter, SLC6A8.

The first disorder identified was GAMT deficiency (OMIM 601240), in 1994, and seven years later, SLC6A8 (OMIM 300352) and AGAT (OMIM 602360) deficiencies were reported [4,5,6]. The hallmarks are the absence/reduction of the creatine signal in the proton magnetic resonance spectroscopy (MRS) of brain, severe delay in expressive speech and language development, and mental retardation. Patients with GAMT deficiency often exhibit a more complex phenotype that includes movement disorder and epilepsy that is sometimes unresponsive to treatment [7,8].

Because of the involvement of creatine and phosphocreatine in muscle metabolism, it could be expected that patients with deficiencies in proteins involved in creatine synthesis or transport would present with muscle problems. However, these patients do not have signs of cardiac myopathy nor do they have pronounced signs of skeletal myopathy. The clinical phenotype mainly involves central nervous system. This suggests that creatine has an additional role in the brain.

Cerebral creatine deficiency syndromes can be diagnosed by measuring creatine levels in brain, which is possible by *in vivo* proton MRS. However to elucidate the underlying defect further biochemical and molecular studies are needed. Creatine and guanidinoacetic acid can be quantified in body fluids [9,10]. In GAMT deficiency, increased levels of guanidinoacetic acid are found in body fluids, whereas reduced levels are found in AGAT deficiency. Creatine is usually reduced in body fluids of patients with a biosynthesis defect, whereas an increased urinary creatine/creatinine ratio is found in males affected with SLC6A8 deficiency. Molecular analysis is available for the three genes encoding AGAT, GAMT and SLC6A8 [11].

Secondary disturbances of creatine metabolism

Secondary creatine metabolism disturbances have been found in patients with defects in urea or glycine metabolism and/or the cofactors of the GAMT enzyme.

The highest concentrations of creatine and phosphocreatine and the highest creatine kinase activities are found in skeletal muscle, hence a close relationship between disturbances of creatine metabolism and various muscle diseases is expected. Many (neuro) muscular diseases, with different underlying defects, display disturbances in creatine metabolism (e.g. Duchenne muscular dystrophy, Becker muscular dystrophy and myotonic dystrophy) [2]. Some of the common findings are increased creatine

concentrations in plasma and urine, decreased urinary creatinine excretion, and depressed muscle levels of creatine and phosphocreatine.

Patients with chronic renal failure commonly present with muscle weakness and disturbances in creatine metabolism. In skeletal muscle of uremic patients the concentrations of phosphocreatine and ATP are decreased, whereas creatine concentrations may be normal or increased [12].

Patients affected with urea cycle disorders present with disturbances in creatine metabolism as well. Guanidinoacetic acid levels were reported to be low in patients with ornithine transcarbamylase deficiency, argininosuccinate lyase deficiency, argininosuccinate lyase deficiency and HHH syndrome (hyperornithinemia, hyperammonemia and homocitrullinuria) [13], however they are normalized with arginine (citrulline) supplementation. This indicates that guanidinoacetic acid is a parameter that should be considered in the follow-up of patients with urea cycle disorders [13,14].

In gyrate atrophy of the choroid and retina, disturbances of creatine metabolism are also observed. The underlying primary defect is a deficiency in mitochondrial matrix L-ornithine:2-oxo-acid aminotransferase, the major enzyme catabolizing ornithine. This leads to an accumulation of ornithine that inhibits AGAT, resulting in decreased formation of guanidinoacetic acid and creatine. As a result, in these patients guanidinoacetic acid and creatine are reduced in both plasma and urine [15,16,17].

Methylation is an important biological process in creatine biosynthesis. Most labile methyl groups are used by the GAMT reaction. Labile methyl groups are generated through a series of reactions that convert methionine to homocysteine. Deprivation of labile methyl groups leads to a functional impairment of the creatine biosynthetic pathway (e.g. combined methylmalonic aciduria and homocystinuria) [18].

OUTLINE of the THESIS

The questions addressed in this thesis involve the diagnosis of CCDS, both at the biochemical and molecular level, as well as their frequency. Furthermore we investigated additional roles for creatine in brain.

As mentioned above, CCDS have as major clinical symptoms mental retardation and absence of expressive speech. Hence, these disorders are very difficult to identify on clinical grounds. To overcome this, we developed and implemented a robust biochemical screening that allows quantification of creatine and guanidinoacetic acid in a single analysis. In addition we established reference values for these compounds in body fluids (**Chapter two**).

Molecular analysis is available for the diagnosis of these disorders. However, if a novel variant in either *AGAT*, *GAMT* or *SLC6A8* gene is found, further work-up is needed to classify it as a pathogenic mutation or a rare variant. We therefore tested if GAMT activity could be restored in GAMT deficient fibroblasts (**Chapter three**) and subsequently developed an approach to study the relevance of variants of unknown significance. Overexpression studies for the *GAMT* gene were performed in transient transfected HeLa cells. This approach was used to test the nature of a common missense mutation in GAMT deficient patients, c.59G>C, p.Trp20Ser (**Chapter four**). Furthermore, we studied the carrier rate of c.59G>C, p.Trp20Ser GAMT mutation in Portugal.

SLC6A8 deficiency - an X-linked disorder- appears to be more common than the two other creatine biosynthesis disorders. In **chapter five** we studied the prevalence of creatine transporter (SLC6A8) deficiency by DNA sequence analysis in an X-linked mental retardation panel (n=290) of the European XLMR Consortium.

In patients affected with CCDS, the most affected organ is the brain. It is known that creatine plays a role in brain energy metabolism. In **chapter six** we tested whether creatine could have additional roles in brain.

The latest developments in the CCDS field are presented in **chapter seven**. It provides an overview of the three disorders, together with diagnostic approaches and a clinical update from the patients reported so far. The novel role of creatine as a

neuromodulator is explored, and finally a projection of what the future could hold for CCDS is also discussed.

In **chapter eight** a summary of the studies described in this thesis is presented together with a brief discussion of the main findings reported. Insights in how the creatine field might be in the near future are also depicted.

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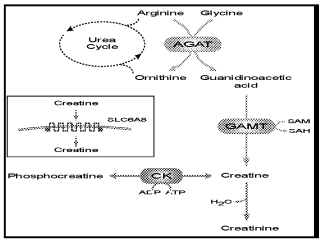
Arginine
Glycine
Urea Cycle
AGAT
Ornithine
Guanidinoacetic acid
Creatine
SLC6A8
Creatine
GAMT
SAM
SAH
Phosphocreatine
CK
ADP ATP
Creatine
H_2O
Creatinine

Chapter 2

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Creatine and guanidinoacetate: diagnostic markers for inborn errors in creatine biosynthesis and transport

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Abstract

In this study, measurements of guanidinoacetate (GAA) and creatine (Cr) in urine, plasma, and cerebrospinal fluid (CSF) were performed using stable isotope dilution gas chromatography–mass spectrometry. Both compounds were analyzed in a single analysis. Reference values were established for GAA and Cr. These values were age dependent. No differences with gender were observed. Eight guanidinoacetate methyltransferase (GAMT) deficient patients and eight creatine transporter SLC6A8 deficient patients were investigated. In urine, plasma, and CSF of GAMT deficient patients increased levels of GAA are present. The SLC6A8 deficient patients all show increased creatine/creatinine (Cr/Crn) ratio in urine demonstrating the importance of the Cr/Crn ratio as a pathognomonic marker of the SLC6A8 deficiency.

Keywords: Guanidinoacetate; Creatine; Creatinine; Creatine transporter; Guanidinoacetate methyltransferase; Arginine:glycine amidinotransferase; Reference values

Introduction

Primary creatine deficiency syndromes (CDS) are a new group of disorders caused by defects in biosynthesis or transport of creatine. Arginine:glycine amidinotransferase (AGAT) deficiency (OMIM 602360) affects the first enzymatic step of creatine biosynthesis [1,2]. Guanidinoacetate methyltransferase (GAMT) deficiency (OMIM 601240) affects the second step. The latter was the first inherited metabolic disorder of creatine metabolism to be recognized [3]. The transport of creatine can be affected due to a deficiency of the creatine transporter (SLC6A8, OMIM 300036) [4], an X-linked trait.

Patients with CDS present with mental retardation, expressive speech and language delay, and epilepsy (varying from intractable seizures in GAMT deficient patients to mild febrile or epileptic seizures in AGAT deficient and transporter deficient patients). GAMT deficient and transporter deficient patients may show autistic behavior; in GAMT deficient patients with a severe phenotype (extra) pyramidal symptoms are present [5,6] (Table 1). The common denominator of these disorders is the depletion of the cerebral creatine pool. In AGAT and GAMT deficiencies the creatine pool can be partially reverted by creatine supplementation. Male patients with the X-linked creatine transporter defect are unresponsive to this supplementation.

Creatine (Cr) and guanidinoacetate (GAA) levels in body fluids (plasma, urine, and CSF), are informative for biochemical diagnosis of GAMT (and possibly also AGAT) deficiency. Biochemical diagnosis for the creatine transporter deficiency is still not clear, however increased urinary levels of Cr have been reported.

Several analytical techniques have been described for the determination of both GAA and Cr in relatively small control populations. Quantitative determination of GAA has been performed by liquid chromatography (LC) and post column derivatization with ninhydrin [7],

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Table 1
Clinical symptoms and biochemical characteristics in disorders of creatine metabolism

	GAMT deficiency	AGAT deficiency	Cr transporter deficiency
Clinical traits			
Mental retardation	+	+	+
Speech and language delay	+	+	+
Epilepsy	+	+	+
(Extra) pyramidal signs	+	−	−
Autistic behavior	+	−	+
H-MRS of brain			
Absence/reduction of Cr	+	+	+
Biochemical findings[a]			
Guanidino acetic acid	↑↑	↓	n
Creatine	↓ (only in plasma)	n-↓	n-↑ (only in urine)
Creatinine	↓	n-↓	n-↓

[a] -, in urine, plasma, and CSF, unless when specifically stated; presence (+), absence (−); increased (↑), decreased (↓), normal (n).

gas chromatography–mass spectrometry (GC–MS) [8], stable isotope dilution (SID) GC–MS [9], high-performance liquid chromatography (HPLC) [10], and isotope dilution electrospray tandem mass spectrometry [11]. However, no extensive studies for the determination of reference values have been performed. We therefore determined the reference values of GAA and Cr in body fluids in a Dutch control population ($n = 225$) of age 0–90 years by a modified SID GC–MS previously developed in our laboratory. Furthermore, eight GAMT deficient patients and eight SLC6A8 deficient patients were investigated.

Materials and methods

Sample collection

Control values were determined in urine, plasma, and CSF samples of individuals (age range: newborns to 90 years) who had no metabolic, renal or neurological disorder. Samples were maintained at −25 °C in the dark until analysis.

Intra assay variation of the metabolite excretion was studied. Random urine samples were collected three times during the day (morning, afternoon, and evening) from six normal individuals (three males and three females, age range 24–56 years). Stability of the compounds due to freezing and thawing was also studied in plasma and CSF samples that were thawed fives times.

The effects of age and gender on GAA and Cr concentrations were investigated. Statistical analysis was performed using the MedCalc software.

Patients and patient's material

Plasma, urine, and/or CSF of eight GAMT-deficient (patients 4–8 unpublished; patients 1–3 previously described [12,13]) and eight SLC6A8-deficient patients (patient 8 unpublished; patients 1–7 published [5]) were included in this study. The diagnosis of these patients was confirmed by enzymatic and molecular studies, and are described elsewhere [5,12,13].

Chemicals

Hexafluoroacetylacetone was obtained from Sigma (St. Louis, MO); pentafluorobenzylbromide was purchased from Pierce (Rockford); guanidinoacetate was obtained from Aldrich (Milwaukee) and labeled GAA was manufactured in the Metabolic Unit, VU Medical Center (Dr. H.J. ten Brink); creatine was obtained from Fluka and labeled creatine from C/D/N Isotopes (Quebec, Canada). All other solvents and chemicals were of analytical grade.

Sample preparation

Determination of GAA and Cr by SID GC–MS was performed according to the method described previously by Struys et al. [9]. The method was adjusted in order to measure both compounds in the same run. Briefly, 100 μl of plasma or CSF, or 50 μl of urine (to which 50 μl of water was added) were used. To 100 μl sample, 50 μl of saturated aqueous sodium bicarbonate, 50 μl hexafluoroacetylacetone, 500 μl toluene and 50 μl internal standard ([$^{13}C_2$]GAA, 0.005, 0.0005, and 0.25 mM solutions for plasma, CSF, and urine; methyl-D3-creatine, 0.05 and 0.5 mM solutions for plasma/CSF and urine, respectively) were added. The mixture was heated to 80 °C for 2 h under continuous stirring and allowed to cool. From the upper toluene phase 300 μl were transferred to another test tube and blown to dryness under nitrogen. Subsequently, 10 μl triethylamine and 100 μl 7% pentafluorobenzylbromide in acetonitrile (v/v) was added. Derivatization was carried out at room temperature for

15 min. Reaction was stopped by adding 200 µl 0.5 N HCl and extraction performed with 1 ml hexane. The hexane layer was finally transferred to a new vial and analyzed.

For both GAA and Cr measurements, calibration curves were established using various amounts of GAA and Cr (plasma: 0.5–10 nmol Cr and 0.05–0.5 nmol GAA; CSF: 0.5–10 nmol Cr and 0.01–0.1 GAA; urine: 5–100 nmol Cr and 5–50 nmol GAA) and constant amounts of internal standard (plasma: 2.5 nmol methyl-D3-creatine and 0.25 nmol $^{13}C_2$-GAA; CSF: 2.5 nmol methyl-D3-creatine and 0.025 nmol [$^{13}C_2$]GAA; urine: 25 nmol methyl-D3-creatine and 12.5 nmol [$^{13}C_2$]GAA). The ratios of standards to internal standard were used for linear regression analysis, and the concentration of the compounds in a sample was obtained by interpolation of the observed ratio.

Compound concentration was expressed as mmol/mol creatinine in urine samples and micromolar for plasma and CSF. Creatinine measurement in urine was performed with the Jaffé method using an automated analyzer, routinely used for clinical chemistry measurements.

Gas chromatography and mass spectrometry conditions

Conditions for the SID GC–MS are described elsewhere [8]. Briefly, for the simultaneous measurement of GAA and Cr, samples were injected splitless at a temperature of 300 °C. Initial oven temperature was maintained at 100 °C for 1 min followed by a ramp of 15 °C min^{-1} to 260 °C. The temperature of the transfer line was set at 300 °C. The column (SGE BPX-70, Bester, Amstelveen, The Netherlands) was inserted directly into the ion source, which was set at 250 °C. Quadrupole temperature was 150 °C. Ammonia was used as moderating gas at an optimized gas pressure. The mass spectrometer (Hewlett–Packard, type Engine) was operated under electron capture negative chemical ionization in the single ion-monitoring mode. The negative ion measured for GAA was m/z −288.13, and for [$^{13}C_2$]GAA was m/z −290.13. The negative ion measured for Cr was m/z −302 and for *N*-methyl-D3-creatine was m/z −305.

Results

Inter/intra assay variations, limit of detection and quantification, stability of the compounds

Intra-assay variability was established by analyzing one sample 10 times. Inter-assay variability was determined by processing the same sample in 10 independent preparations on different days (see Table 2).

The lower limit of detection of GAA (S/N = 5) was 0.01 µM whereas the lower limit of quantification (S/N = 10) is 0.02 µM. The lower limit of detection for Cr (S/N = 5) was 0.0012 µM; the lower limit of quantification is 0.0024 µM.

Plasma and CSF samples were thawed five times and no effect was observed on compound concentration.

Reference values of guanidino acetate and creatine

In urine, the GAA values ranged from 4 to 220 mmol/mol creatinine (Table 3), and from 6 to 1208 mmol/mol creatinine for Cr (Table 4).

In plasma and CSF, GAA values ranged from 0.35 to 3.5 µM and from 0.036 to 0.22 µM respectively (Table 3). Creatine, values ranged from 6 to 109 µM and from 24 to 66 µM in plasma and CSF respectively (Table 4).

No significant difference between gender was observed (P(GAA) = 0.062; P(Cr) = 0.261). GAA and Cr, in urine and plasma were found to be age dependent (P = 0.001).

Biological individual variation of guanidinoacetate and creatine

Individual biological variation of the metabolites was assessed in six control individuals. No significant difference (P = 0.654) was found between the three samples tested for each individual (data not shown).

Patients

Eight GAMT and eight SLC6A8 deficient patients are included in this study.

All GAMT deficient patients showed increased levels of GAA in urine (Table 3). In plasma of patients 1–3 and

Table 2
Intra- and inter-assay variability of the SID GC–MS method

	Intra-assay SID GC–MS (n = 10)		Inter-assay SID GC–MS (n = 10)	
	GAA (µM)	Cr (µM)	GAA (µM)	Cr (µM)
Urine	36 ± 0.80	198 ± 15	39 ± 2.0	208 ± 19
	CV = 2.0%	CV = 8.0%	CV = 5.0%	CV = 9.0%
Plasma	2.0 ± 0.07	46 ± 2	2.2 ± 0.10	45 ± 1.7
	CV = 4.0%	CV = 4.0%	CV = 4.5%	CV = 3.8%
CSF	0.25 ± 0.02	57 ± 3.4	0.25 ± 0.01 (n = 5)	62 ± 3.7 (n = 5)
	CV = 6.0%	CV = 6.0%	CV = 4.0%	CV = 6.0%

Table 3
GAA as a marker of GAMT deficiency

Reference values	Urine ($n = 140$)		Plasma ($n = 60$)		CSF ($n = 25$)	
GAA (μM)	0–15 years	9–1142	0–15 years	0.35–1.8	All ages	0.02–0.56
	>15 years	20–577	>15 years	1.0–3.5		
GAA (mmol/mol creatinine)	0–15 years	4–220				
	>15 years	3–78				
GAMT deficient patients	Urine (mmol/mol creatinine)		Plasma (μM)		CSF (μM)	
	GAA	Cr	GAA	Cr		
Pt1 (3 yr)	4368 ↑	276	39 ↑	5 ↓	14 ↑	
Pt2 (5 yr)	624 ↑	22	20 ↑	2↓	15 ↑	
Pt3 (25 yr)	612 ↑	35	12 ↑	1 ↓	na	
Pt4 (2 yr)	2809 ↑	336	na	na	na	
Pt5 (20 yr)	691 ↑	51	na	na	na	
Pt6 (17 yr)	1005 ↑	35	na	na	na	
Pt7 (20 yr)	529 ↑	48	na	na	na	
Pt8 (22 yr)	490 ↑	19	na	na	na	

na, not available.

Table 4
Cr/Crn ratio as a marker of SLC6A8 deficiency

Reference values	Urine ($n = 140$)		Plasma ($n = 60$)		CSF ($n = 25$)	
Cr (μM)	<4 years	5–6725	<10 years	17–109	All ages	17–87
	4–12 years	36–4964	>10 years	6–50		
	>12 years	31–2588				
Cr/Crn ratio	<4 years	0.006–1.2				
	4–12 years	0.017–0.72				
	>12 years	0.011–0.24				
SLC6A8 deficient patients	Urine		Plasma (μM)	CSF (μM)		
	Cr/Crn ratio	Cr (μM)	Cr	Cr		
Pt1 (7 yr)	1.5 ↑	2185	75	62		
Pt2 (20 yr)	3.1 ↑	8570 ↑	92 ↑	56		
Pt3 (4 yr)	3.1 ↑	17254 ↑	68	na		
Pt4 (2 yr)	5.5 ↑	2684	96	na		
Pt5 (4 yr)	4.5 ↑	na	83	na		
Pt6 (66 yr)	2.5 ↑	na	na	na		
Pt7 (2 yr)	3.8 ↑	na	69	na		
Pt8 (5 yr)	1.4 ↑	7838 ↑	na	na		

na, not available.

CSF of patients 1 and 2 GAA levels are also increased (Table 3). In plasma Cr was decreased while in urine it was not significantly different from controls. In three out of five SLC6A8 deficient patients (mildly) increased levels of Cr (expressed as μM) in urine were found. However, all the eight patients showed increased Cr/Crn ratio (Table 4). In plasma of patient 2, Cr levels were increased. GAA levels were within normal range in both plasma and urine (data not shown).

Discussion

Inborn errors of creatine metabolism (biosynthesis and transport) can be recognized by the marked reduction of the creatine signal in proton magnetic resonance spectra (MRS) of the brain. However, metabolite screening and molecular analysis remain necessary to unravel the underlying defect. Moreover, MRS of brain is not available in most institutes, and is therefore not a candidate method to screen a large group of patients. The present paper describes and illustrates the value of metabolite screening using SID GC–MS.

SID GC–MS is a very accurate method, enabling selective and sensitive measurements due to specific derivatization (for details see [9]). Furthermore, it allows the analysis of GAA and Cr in a single run. Variation of the compounds during the day was not significant, indicating that a random urine sample is sufficient for the diagnosis of CDS. Indeed in all cases, so far, we were able to identify the individual metabolic defect in CDS based on a random urine sample. Additionally, we found in the reference values a correlation of GAA and Cr with age. Our reference values for GAA and Cr are in the same range as those described previously. Carducci et al. [10] presented age-related reference values for both

compounds, but direct comparison of the creatine reference values is impossible as the sum of creatine and creatinine is reported. However, when we calculate the sum of Cr and Crn in our samples, the values are comparable. In addition, reference values for these compounds were also reported by Marescau et al. [7] and Bodamer et al. [11]. In these studies no age-related values are presented and the age range studied is smaller, as only adults values are presented. Our values are also comparable to theirs.

Increased GAA is a valid marker for the identification of GAMT deficiency. The GAA levels of our patients were 4–130 times increased in urine (when expressed per creatinine) and 4–22 times in plasma. In AGAT patients it was reported that GAA in plasma was decreased [2,10]. In the last decade only this one family with AGAT deficiency was identified (in comparison with more than 10 families affected with GAMT deficiency). AGAT deficient patients could remain unnoticed in screening studies, due to the use of techniques that are not sensitive or specific enough to detect decreased metabolite levels.

The biochemical diagnosis of the creatine transporter deficiency is more complicated compared to several metabolic disorders, where typically accumulation of a substrate occurs. In the creatine transporter patients (see Table 4) three out of five patients had mildly increased (2–3 times) excretion of Cr (expressed in micromolar). However, the Cr/Crn ratio was increased in eight out of eight identified patients (2–13 times compared to the highest value found in controls). Thus, currently the Cr/Crn ratio represents the most valuable biochemical marker for SLC6A8 deficiency. The disturbed ratio observed in the SLC6A8 deficient patients is probably due to: (1) a dysfunctional Cr transporter, which is not able to take up Cr into cells resulting in an increased excretion in urine; (2) decreased intracellular Cr, which results in decreased Crn since only intracellular Cr is converted to creatinine. The Cr/Crn ratio in the SLC6A8-deficient patients may be underestimated by ca. 10%. This is caused by the interference of Cr in the Crn determination by the Jaffé method, which results in maximal 10% higher Crn values (results not shown). Assessment of Cr plus Crn has been performed using a HPLC method [10]. We, however, would be cautious to use the combined Cr + Crn value for the screening of creatine transporter deficiency. The combined Cr + Crn value may mask the individual differences seen in each parameter (i.e., Cr is mildly increased and Crn mildly decreased), which may result in values, that are not different from those found in controls.

The present paper provides reference values of metabolites in creatine metabolism (GAA, Cr, and Cr/Crn ratio) for different age groups, and illustrates its pivotal importance in the screening and detection of CDS patients. This biochemical approach is applicable for the screening of large group of patients with clinical features suggestive of CDS (e.g., mentally retarded patients). In our opinion, this biochemical approach should be offered in a diagnostic setting for patients with unexplained mental retardation, dysphasia, epilepsy, and/or autistic behavior. Follow-up investigations by molecular analysis of the gene and/or functional tests (AGAT, GAMT activities, or creatine uptake) are warranted for a definite diagnosis.

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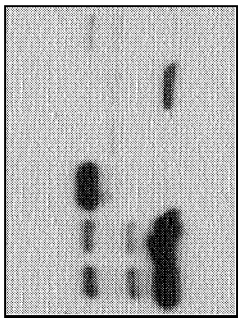

Chapter 3

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Brief Communication

Overexpression of GAMT restores GAMT activity in primary GAMT-deficient fibroblasts

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Abstract

Guanidinoacetate methyltransferase deficiency (MIM 601240) is an autosomal recessive disorder of creatine biosynthesis. Patients present with mental retardation, extrapyramidal symptoms, autistic-like behavior, epilepsy, cerebral creatine deficiency and increased levels of guanidinoacetate. So far 15 mutations have been reported, including six missense variants that are highly likely to be pathogenic mutations. To prove that mutations in the *GAMT* gene are responsible for GAMT deficiency we overexpressed the GAMT open reading frame in GAMT-deficient fibroblasts by stable transfection. In addition, HeLa cells were transiently transfected with the same expression vector. In contrast to mock transfectants transfection of primary GAMT-deficient fibroblasts with wild-type GAMT results in the restoration of GAMT activity as measured by GC–MS using stable isotope labeled substrates. Moreover, the expression of the GAMT–EGFP fusion protein was analyzed by Western blot, confirming the presence of GAMT fusion protein, both in the stable as well as in the transient transfectants. Here, we prove that mutations in the *GAMT* gene are responsible for GAMT deficiency, since overexpression of the GAMT open reading frame restores GAMT activity in GAMT-deficient fibroblasts. Furthermore, the transient transfection of HeLa cells will be important for functional analysis of variants of unknown consequence (i.e., missense mutations).

Keywords: Cerebral creatine deficiency syndrome; GAMT; Mental retardation; Transfection

Introduction

Guanidinoacetate methyltransferase (GAMT) deficiency (MIM 601240), the first identified error of the creatine metabolic pathway, is an autosomal recessive disorder of creatine biosynthesis [1]. The disease usually manifests with developmental delay at the first months of life. Neurological symptoms are heterogeneous and may include muscular hypotonia and weakness, progressive extrapyramidal signs and symptoms, mental retardation, epilepsy and autistic or self-aggressive behavior in older patients [1,2]. Phenotypically, GAMT-deficient patients can be classified as having a mild, intermediate or severe phenotype according to the severity of the main features of the deficiency (mental retardation, epilepsy and motor handicap) [3]. Biochemical findings include cerebral creatine deficiency, accumulation of guanidinoacetate and low creatine levels in body fluids [1,4,5]. Confirmation of the diagnosis is made by mutational analysis and enzyme assays [1,6,7]. Treatment with creatine and ornithine supplementation (combined with arginine restriction) results in clinical improvement and restoration of the cerebral creatine pool (≈70%) [8]. So far, there are 29 patients reported and 15 mutations in the *GAMT* gene (GeneID 2593) have been described [3,9,10].

In order to provide final proof that GAMT deficiency is caused by a defect of the GAMT protein, the GAMT open reading frame (ORF) was stable transfected into primary fibroblasts of a GAMT-deficient patient, and showed restoration of GAMT activity.

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Methods

Overexpression of the GAMT ORF in GAMT-deficient fibroblasts

The ORF of the *GAMT* gene (NM_000156) was amplified by PCR using cDNA from a control fibroblast cell line. The PCR product (with restriction sites added for *Hin*dIII and *Bgl*II) was cloned into pCR®2.1-TOPO® (Invitrogen) and verified by DNA sequencing (ABI3100). The ORF was excised from this plasmid by digestion with *Hin*dIII and *Bgl*II and cloned in frame with Enhanced Green Fluorescent Protein in an expression vector (pEGFP-N1 Clonetech) predigested with *Hin*dIII and *Bam*HI. This construct was then stably transfected into primary fibroblasts of a GAMT-deficient patient or transient transfected in HeLa cells. The GAMT-deficient fibroblasts contain a common homozygous missense mutation (c.59G > C; p.Trp20Ser) [3]. Stable transfections were performed using 25 μg of the above described expression vector (pEGFP–GAMT) using polyethyleneimine (PEI, Polysciences, Omnilabo) and selected with G418. Transient transfections were performed as above, except that the cells were additionally co-transfected with 2.5 μg of pBABE-Puro to enable selection with puromycin. The cultures were maintained in HAMF10 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and kept under selection with either G418 (10 μg/ml) or puromycin (0.5 μg/ml). The transfection experiments were performed in triplicate. Pellets were stored for further use (i.e., GAMT assay and Western blot analysis) at −80 °C.

The GAMT enzyme assay was performed according to Verhoeven et al. [7]. Briefly, fibroblast lysates were incubated with substrates labeled with [$^{13}C_2$]-guanidinoacetate and [$^{2}H_3$]-*S*-adenosylmethionine. After incubation with these substrates the reaction product [$^{13}C_2$–$^{2}H_3$]-creatine was analyzed by gas chromatography–mass spectrometry using $^{2}H_3$-creatine as internal standard.

To detect the GAMT–EGFP fusion protein expression, pellets were lysed and protein concentration was determined using the bicinchoninic acid method. Equal amounts of protein (30 μg) were separated in a 12% SDS–polyacrylamide gel and transferred to a Hybond™ membrane by standard procedures. Immunodetection was performed using an EGFP antibody and ECL detection kit (Amersham Biosciences).

Results

Overexpression of the GAMT–EGFP fusion protein restores GAMT activity

Stable transfection of primary GAMT-deficient fibroblasts with pEGFP–GAMT results in the restoration of GAMT activity (501 pmol creatine/mg protein/h), in contrast to mock transfectants (1 pmol creatine/mg protein/h) (Fig. 1a). A 56-fold increased GAMT activity was detected in transient transfected HeLa cells (7700 pmol creatine/mg protein/h), compared to that of mock transfected HeLa cells (137 pmol creatine/mg protein/h) (Fig. 1b). GAMT activities in normal fibroblasts range from 60 to 243 pmol creatine/mg protein/h. To prove proper expression of the GAMT protein, cell extracts from the pEGFP–GAMT transfected fibroblasts and HeLa cells, as well as that of the mock transfectants, were analyzed by Western blot using an anti EGFP antibody. Western blot analysis showed the presence of the GAMT–EGFP fusion protein of approximately 60 kDa in the

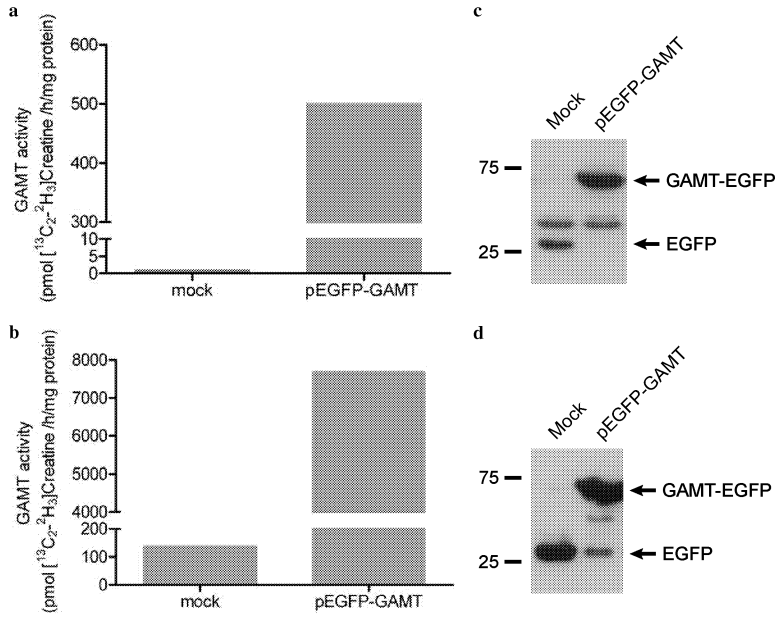

Fig. 1. Overexpression of GAMT–EGFP restores GAMT protein expression and activity in cells. (a) GAMT activity in primary GAMT-deficient fibroblasts stable transfected with pEGFP (mock) or with pEGFP–GAMT, (b) GAMT activity in HeLa cells transient transfected with pEGFP (mock) or with pEGFP–GAMT, (c) Western blot analysis of cell extracts from GAMT deficient fibroblasts expressing EGFP (mock) or GAMT–EGFP (pEGFP–GAMT) (fusion) protein, (d) Western blot analysis of extracts of cell extracts from HeLa cells expressing EGFP (mock) or GAMT–EGFP (pEGFP–GAMT). Equal amounts of protein were loaded (30 μg).

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pEGFP–GAMT transfectants (Fig. 1c and d). This in contrast to the mock transfectants (empty vector) where only the EGFP of approximately 30 kDa was detected.

Discussion

In the present work, definitive proof is provided that mutations in the *GAMT* gene are responsible for GAMT deficiency, a cerebral creatine deficiency syndrome. This was achieved by stable transfections of primary GAMT deficient fibroblasts with a GAMT–EGFP construct resulting in the restoration of the GAMT protein expression and activity. Recently, the same approach was used for the *SLC6A8* gene. Mutations in this gene are the primary cause of creatine transporter deficiency, another cerebral creatine deficiency syndrome [10].

The advantage of stable transfections is that only a few copies of the vector will be integrated in the genomic DNA, resulting in lower protein expression, similar to the physiological condition. However, the disadvantage is that it is time consuming (~3–6 months), since primary fibroblasts are slow dividing cells, which are difficult to transfect. Stable transfections—especially in case of non-transformed fibroblasts—are laborious and not easy to use in a diagnostic setting. However, in case that fast growing cells (e.g., HeLa cells) are used, results can be obtained in one week. Therefore, HeLa cells were also transfected with the GAMT–EGFP construct. Although HeLa cells have a basal endogenous GAMT activity, the overexpression of the GAMT construct resulted in a significant increase in GAMT activity in comparison to the endogenous levels. Even though the high levels of GAMT overexpression do not represent physiological conditions, this would not be inconvenient in a diagnostic setting, where the main question is whether a mutation in the GAMT coding sequence has an effect on its enzyme activity or not. Hence, we propose that transient transfections of HeLa cells are most suitable for diagnostic purposes. The assay will be based on the comparison of GAMT activity of transient transfectants containing either the wild-type GAMT-ORF or the variant-GAMT-ORF. In the case that the GAMT activity is similar in these transfectants the variant should be classified a rare variant of unknown significance. However, if the activity is not increased by the overexpression of the variant-GAMT-ORF, the variant can be classified as a proven pathogenic mutation. The approach is also applicable for compound heterozygous mutations. It should be noted, however, that both mutations need to be introduced separately in the pEGFP–GAMT construct, and that both constructs need to be tested individually.

To date, 6 out of 15 reported mutations (40%) in the *GAMT* gene are missense mutations [3,9]. Therefore, an approach to investigate the nature of missense variants in the *GAMT* gene is needed. This method can also be used to classify novel sequence variants in other genes, including the genes involved in cerebral creatine deficiency syndromes *SLC6A8* [11] and *AGAT*. Although in the *AGAT* gene so far no missense mutations have been detected.

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Chapter 4

A PREVALENT PATHOGENIC *GAMT* MUTATION (C.59G>C) IN MEDITERRANEAN REGIONS

L.S. Almeida[1], L. Vilarinho[2], P.S. Darmin[1], E.H. Rosenberg[1], C. Martinez-Muñoz[1], C. Jakobs[1], and G.S. Salomons[1*]

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Running title: c.59G>C mutation in GAMT deficiency

ABSTRACT

Guanidinoacetate methyltransferase (GAMT) deficiency (MIM 601240), an autosomal recessive disorder of creatine biosynthesis, presents with mental retardation, extrapyramidal symptoms, autistic-like behavior and epilepsy. Other hallmarks are cerebral creatine deficiency, increased levels of guanidinoacetate in body fluids and mutations in the *GAMT* gene. Creatine supplementation partially restores cerebral creatine content. Worldwide, 29 patients have been identified and 15 different mutations have been reported in the *GAMT* gene. Ten out of these 29 patients are of Portuguese origin. Likely, a founder effect and a high carrier rate in Portugal exist, since in 17 out of the 20 Portuguese alleles the c.59G>C; p.Trp20Ser mutation was found. We investigated the carrier rate of the c.59G>C; p.Trp20Ser mutation in different regions of Portugal and confirmed the pathogenic nature of this missense mutation by transient transfections. 1002 anonymous bloodspots were screened for the presence of the c.59G>C; p.Trp20Ser mutation by SNaPshot (Single Nucleotide Polymorphism Multiplex Kit). Eight carriers of c.59G>C; p.Trp20Ser were detected of which four are derived from the Archipelagos. This suggests that the carrier rate of the c.59G>C; p.Trp20Ser mutation is relatively high in these islands, as well as in other parts of Portugal. It also implies that newborn screening in these regions is warranted for this treatable disorder.

KEY WORDS: creatine deficiency, GAMT, c.59G>C; p.Trp20Ser, W20S, carrier rate

INTRODUCTION

In 1994 the first disorder of creatine biosynthesis, guanidinoacetate methyltransferase (GAMT) deficiency (MIM 601240) was reported [1,2]. GAMT deficiency is an autosomal recessive disorder, and patients usually present with developmental delay in the first months of life, muscular hypotonia and weakness, progressive extrapyramidal signs and symptoms, mental retardation, epilepsy and autistic or self-aggressive behavior [2,3]. According to the severity of the main clinical features (mental retardation, epilepsy and motor handicap) GAMT deficiency is classified as a mild, intermediate or severe phenotype [4]. Biochemically, patients show cerebral creatine deficiency, accumulation of guanidinoacetate and reduced creatine levels in body fluids [5,6]. Various methods, including stable isotope dilution gas chromatography-mass spectrometry (SID GC-MS), high performance liquid chromatography, and tandem MS are being used to measure these metabolites in body fluids. Furthermore, the metabolites can also be quantitated in dried blood spots by tandem mass spectrometry [7,8,9]. Confirmation of the diagnosis is obtained by mutation detection and enzyme assays [2,10]. Patients with GAMT deficiency, especially if treated early in life, have shown favorable response to oral supplementation of creatine monohydrate and dietary restriction of arginine in combination with ornithine supplementation [11].

So far, 29 patients have been described from a total of 22 families (Figure 1a) [4,11,12] showing a broad clinical spectrum from mild developmental delay and autistic features to severe mental retardation and severe epilepsy. Fifteen mutations in the *GAMT* gene that encodes a protein of 236 amino acids have been reported. Recently, ten Portuguese patients were identified of whom eight are homozygous and one is compound heterozygous for the c.59G>C; p.Trp20Ser (W20S) mutation (4,13), suggesting a high carrier rate for this mutation. We therefore investigated the prevalence of the c.59G>C; p.Trp20Ser mutation in different regions of Portugal. A high prevalence would argue for inclusion of GAMT deficiency in the neonatal screening.

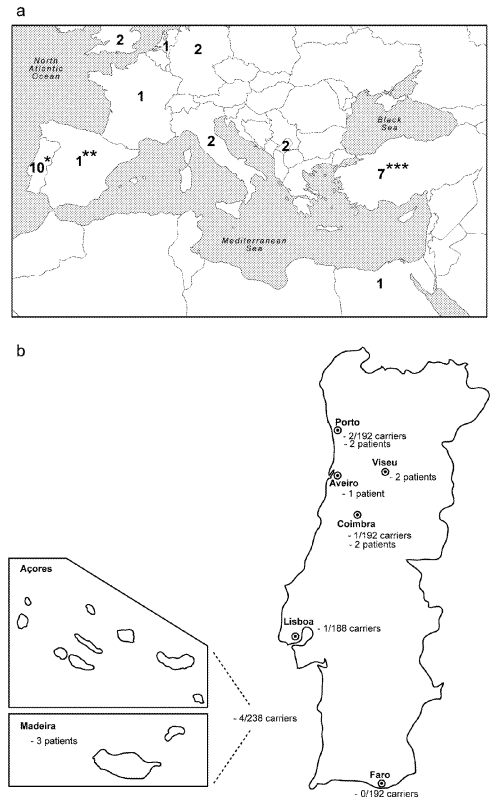

Figure 1. Localization of GAMT deficiency worldwide. A) overview of GAMT deficient patients, that have been described in the literature are depicted per country. * Nine patients carry the c.59G>C; p.Trp20Ser mutation (8 homozygotes and 1 heterozygote) ** In DNA of the patient the heterozygous c.59G>C; p.Trp20Ser mutation was detected. *** In DNA of one of the patients the homozygous c.59G>C; p.Trp20Ser mutation was detected. B) carrier rate of c.59G>C; p.Trp20Ser in Portugal. Carrier rate was determined from 1002 blood spots obtained from the national neonatal screening committee.

MATERIAL AND METHODS

Subjects

Anonymous blood spot samples (1002), from various Portuguese areas were obtained from the Portuguese Neonatal Screening Committee (Porto n=192, Coimbra n=192, Lisboa n=188, Faro n=192, Archipelagos n=238)

Screening for the presence/absence of the c.59G>C; Trp20Ser

DNA was extracted from dried blood spots on filter paper (QIAamp® 96 DNA blood kit, Qiagen). Exon 1 of the *GAMT* gene was amplified by PCR using the following primer pair:

forward primer, 5'-GGCGGCGCGCGATCGAGGTCGGGTCGCCGT-3' and reverse primer, 5'-CGGGGCAGCCCCGGCCTCAGTTTCCCCTGC-3'. Amplification was initiated after 15 min Hotstart T*aq* (Qiagen) activation step at 95^{o}C, followed by 38 cycles of denaturation, annealing and amplification (45 sec at 94^{o}C, 45 sec at 60^{o}C and, 1 min at 72^{o}C). PCR products were purified (Millipore vacufold multiscreen PCR plates) and subsequently analyzed by SNaPshot.

The SNaPshot procedure was performed according to the instructions of the manufacturer (ABIPrism SNaPshot multiplex kit, Applied Biosystems) using a primer (5'-AGAACTGCAGCCCCGCGT-3') directly upstream of the nucleotide being involved in the mutation (c.59G>C; p.Trp20Ser). Calf intestinal phosphatase (CIP, 1U/µl) was used to remove excess of dNTP, ddNTP and primers. The SNaPshot reactions were resolved on a genetic analyzer in the GeneScan mode (ABI3100). In case a heterozygous mutation was detected by SNaPshot its presence was confirmed by direct DNA sequence analysis of an independent PCR product.

Transient transfection of GAMT-W20S ORF in HeLa cells

The open reading frame (ORF) of the *GAMT* gene was cloned as described previously [14]. In brief, the GAMT ORF containing the c.59G>C; p.Trp20Ser mutation was amplified by PCR using cDNA from a GAMT deficient fibroblast cell line containing the homozygous mutation with forward primer 5'-CGCGCGATCGAGGTCGGGTC-3' and reverse primer 5'-GAAGCCGGGAAAGCTTCTGGTG-3'. The PCR product was cloned in frame with Enhanced Green Fluorescent Protein (EGFP) in expression vector pEGFP-N1 (Clonetech). This construct (pEGFP-W20S), as well as, the empty vector (pEGFP-N1) and pEGFP-GAMT were then transiently transfected into HeLa cells as described previously (Almeida et al, 2006). Cell pellets for GAMT activity and Western blot were stored at -80ºC until further use.

The GAMT enzyme assay was performed according to Verhoeven et al 2004. To prove GAMT-EGFP fusion protein expression, all lysates of the transfectants (30 µg) were separated in a 12% SDS-polyacrylamide gel and transferred to a Hybond™ membrane by standard procedures. Immunodetection was performed using an anti-EGFP antibody and ECL detection kit (Amersham Biosciences).

RESULTS

Validation of the SNP detection by SNaPshot

To ascertain that the SNaPshot procedure allows the detection of wild type c.59G, homozygous or heterozygous c.59G>C, the procedure was tested using the DNA isolated from a control, a

GAMT deficient patient (c.59G>C; p.Trp20Ser homozygous) and a carrier of the mutation (c.59G>C; p.Trp20Ser heterozygous). As illustrated in figure 2 the three genotypes are easily discriminated using SNaPshot.

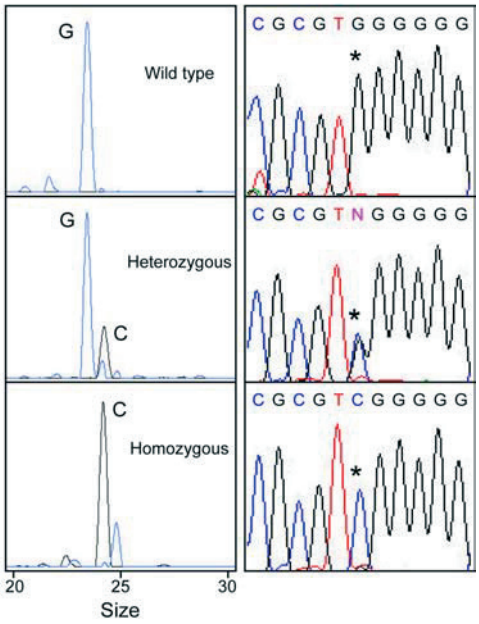

Figure 2. Detection of c.59G>C; p.Trp20Ser mutation using SNaPshot. Left panels illustrate the three electropherograms obtained using SNaPshot; right panels illustrate electropherograms of direct DNA sequencing confirmations.

Carrier Rate of c.59G>C; p.Trp20Ser

Blood spots from different regions of Portugal (Figure 1b), were screened for the c.59G>C; p.Trp20Ser mutation using SNaPshot. Eight heterozygous subjects were detected, suggesting a carrier rate of 0.8% (CI 0.2-1.3%). The highest frequency of heterozygous individuals was detected in the Archipelagos (Madeira and Açores islands, 4/238) and in Porto (2/192). In Coimbra and Lisboa one carrier was identified respectively (1/192 and 1/188). In the Archipelagos the carrier rate may be as high as 1.7% (Table 1).

Table 1. Carrier rate of the c.59G>C; p.Trp20Ser mutation in Portugal

Location	Number tested	Number of carriers	%	CI
Archipelagos	238	4	1.7	0-3.3
Porto	192	2	1.0	0-2.5
Coimbra	192	1	0.5	0-1.5
Lisboa	188	1	0.5	0-1.6
Faro	192	0		
Cumulative	1002	8	0.8	0.2-1.3

The p.Trp20Ser allele does not increase GAMT activity in HeLa cells

In order to establish the pathogenicity of the c.59G>C; p.Trp20Ser mutation, HeLa cells were transfected with pEGFP-W20S(Trp20Ser). In contrast to the pEGFP-GAMT transfection, where the GAMT activity did increase (60 fold), the GAMT activity did not increase upon transfection with pEGFP-W20S nor with the pEGFP-N1 (mock).

To investigate proper expression of the GAMT fusion proteins, cell lysates from the pEGFP-W20S, as well as pEGFP-GAMT and pEGFP-N1 transfected HeLa cells were analyzed by SDS-PAGE Western Blot using an anti-EGFP antibody. High levels of the GAMT fusion protein (≈ 60 kDa) were detected in both pEGFP-GAMT and pEGFP-W20S and cells (Figure 3). In the mock transfectant, the EGFP protein (≈ 30kDa) was present (Figure 3, A). This shows that the lack of GAMT activity in the pEGFP-W20S transfection is not due to the absence of the protein expression.

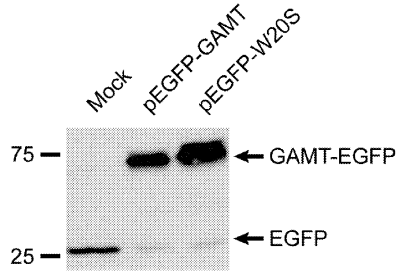

Figure 3. Detection of EGFP (Enhanced Green Flurescent Protein) and GAMT-EGFP fusion protein by Western blotting using anti EGFP antibody. Mock transfectant, only the EGFP protein (≈ 30kDa) is detected whereas in pEGFP-GAMT and pEGFP- W20S(Trp20Ser) transfectants, GAMT-EGFP fusion protein (≈ 60 kDa, see arrow) is detected.

DISCUSSION

In literature, 29 patients affected with GAMT deficiency have been described. Strikingly, among those, ten are of Portuguese origin and 17 of the 20 alleles contain the same mutation, (c.59G>C; p.Trp20Ser) (Figure 1).

This led us to study the prevalence of this mutation among the Portuguese population by SNaPshot in dry bloodspots of Porto, Coimbra, Lisboa, Faro and the Archipelagos. In total, eight carriers were identified out of 1002 individuals, suggesting a carrier rate of 0.8% (CI = 0.2-1.3%). In the Archipelagos and Porto the carrier rate may be as high as 1.7 % (CI = 0-3.3) and 1.0 % (CI = 0-2.5), respectively. In the Archipelagos, that include the Madeira and Açores islands, the population has lived in relative isolation and this may explain the relatively high prevalence compared to other regions in Portugal. However, Porto is a large urban area and also there the prevalence appears to be high. These two regions are geographically distant, suggesting that GAMT deficiency occurs more frequently in Portugal than in other countries due to founder effect. Already a high number of founder mutations, of which several are associated with rare autosomal recessive disorders of metabolism (e.g. the Machado-Joseph disease [15], Gaucher disease [16] argininemia and 3-hydroxy-3-methylglutaric aciduria [17,18], have been detected in Portugal.

The molecular approach that we chose to investigate the presence of the c.59G>C; p.Trp20Ser mutation has several advantages: 1) the method is rapid (data available within 24 hours), of high throughput and has low costs 2) the results are reliable and there is clear discrimination between wild type, homozygous mutation or heterozygous mutation, 3) the method is easy to implement in a diagnostic setting,. A drawback could be that an expensive capillary electrophoresis system is needed for high throughput analysis. However, in many diagnostic laboratories such equipment is used for DNA sequence and/or gene scan analysis and thus might already be available. Furthermore, in case of suspicion of GAMT deficiency (i.e. high guanidinoacetate levels in body fluids or by the almost or complete absence of cerebral creatine) in Mediterranean countries, the SNaPshot analysis may be used to quickly scan for the presence of the c.59G>C; p.Trp20Ser mutation.

The homozygous or compound heterozygous c.59G>C; p.Trp20Ser mutation has been found in 8 and 1 Portuguese patients, respectively. Furthermore compound heterozygosity for the c.59G>C; p.Trp20Ser mutation has been reported in a Spanish patient; the homozygous c.59G>C; p.Trp20Ser has also been found in a Turkish patient. The mutation was considered pathogenic as it affects a highly conserved amino acid residue (Figure 4, see box alignment: conserved in all species except in *Canis familiaris*), it was not found in 210 control chromosomes and it is associated with impaired GAMT activity in fibroblasts. Here, we proved that c.59G>C; p.Trp20Ser is pathogenic since, in pEGFP W20S transient transfected HeLa

cells, increased GAMT activity could not be detected, in contrast to the wild type pEGFP-GAMT transfectants. High expression levels of EGFP-W20S protein were detected in the transfected cells indicating that the transfections were successfull which proves that GAMT W20S has no detectable enzyme activity.

The heterogenic clinical presentation of the GAMT patients has resulted in the classification of mild, intermediate or a severe phenotype. Early diagnosis of this progressive disorder is important as the clinical outcome in GAMT deficient patients appears to be related to the age that the treatment is started [11]. Favorable response to oral supplementation of creatine and ornithine has been shown, but complete reversal of the symptoms has not yet been observed in GAMT deficient patients. Diagnosis of GAMT deficiency is feasible already at birth [11]. Initiation of treatment early in life and perhaps at a presymptomatic stage of the disease will be effective in improving the outcome in patients with GAMT deficiency. Neonatal screening trials for this treatable disorder have been initiated in various countries using blood spots and tandem MS. Unfortunately, no data has been published yet. Neonatal screening at the metabolite level will allow the detection of GAMT deficient patients, who may have novel mutations. In case metabolite screening is not feasible, screening at the molecular level in Portugal for the c.59G>C; p.Trp20Ser mutation and perhaps in other Mediterranean countries, likely will result in the detection of the majority of GAMT deficient patients. So far, 9 out of 10 GAMT deficient patients of Portuguese origin contained at least one c.59G>C; p.Trp20Ser allele. The SNaPshot method as well as other SNP detection assays could be considered, including pyrosequencing or mutation scanning methods like DHPLC [19].

Screening for GAMT deficiency in the Archipelagos and Porto meets the criteria for a population screening 1) high frequency of carriers in the target population, 2) availability of a specific and reliable test, 3) access to genetic counseling to couples identified as carriers, and 4) availability of prenatal diagnosis. The authors suggest implementing neonatal screening in Portugal, especially in the areas where a relative high prevalence c.59G>C; p.Trp20Ser of carrier rate has been shown.

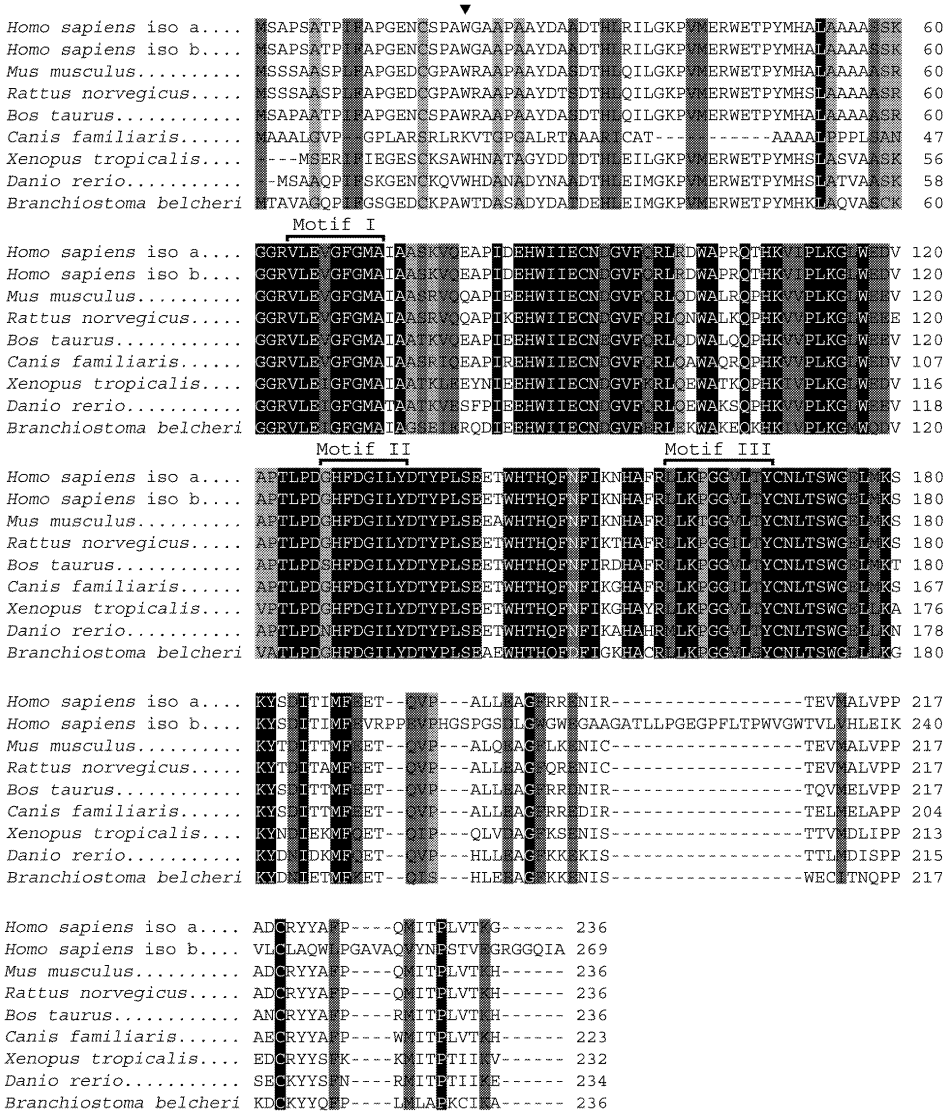

Figure 4. Multiple-sequence alignment among GAMT proteins of different species. The GAMT protein sequence is shown for the following species (identified by Blastp search to be most related to the *Homo sapiens* GAMT protein): *Homo sapiens* isoform a, *Homo sapiens* isoform b, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Canis familiaris*, *Xenopus tropicalis*, *Danio rerio*, *Branchiostoma belcheri*. Alignment was determined by Clustal W; conserved aminoacids are highlighted in black; conserved substitutions are highlighted in grey and semi-conserved substitutions are highlighted in light grey. Trytophan at position 20 is indicated by an arrow head. Note that Trp (W) is conserved among all species except in *Canis familiaris*. Methyltransferase sequence motifs I, II and III are also illustrated [20].

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Chapter 5

Am. J. Hum. Genet. 75:97–105, 2004

Report

High Prevalence of SLC6A8 Deficiency in X-Linked Mental Retardation

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A novel X-linked mental retardation (XLMR) syndrome was recently identified, resulting from creatine deficiency in the brain caused by mutations in the creatine transporter gene, *SLC6A8*. We have studied the prevalence of *SLC6A8* mutations in a panel of 290 patients with nonsyndromic XLMR archived by the European XLMR Consortium. The full-length open reading frame and splice sites of the *SLC6A8* gene were investigated by DNA sequence analysis. Six pathogenic mutations, of which five were novel, were identified in a total of 288 patients with XLMR, showing a prevalence of at least 2.1% (6/288). The novel pathogenic mutations are a nonsense mutation (p.Y317X) and four missense mutations. Three missense mutations (p.G87R, p.P390L, and p.P554L) were concluded to be pathogenic on the basis of conservation, segregation, chemical properties of the residues involved, as well as the absence of these and any other missense mutation in 276 controls. For the p.C337W mutation, additional material was available to biochemically prove (i.e., by increased urinary creatine:creatinine ratio) pathogenicity. In addition, we found nine novel polymorphisms (IVS1+26G→A, IVS7+37G→A, IVS7+87A→G, IVS7-35G→A, IVS12-3C→T, IVS2+88G→C, IVS9-36G→A, IVS12-82G→C, and p.Y498) that were present in the XLMR panel and/or in the control panel. Two missense variants (p.V629I and p.M560V) that were not highly conserved and were not associated with increased creatine:creatinine ratio, one translational silent variant (p.L472), and 10 intervening sequence variants or untranslated region variants (IVS6+9C→T, IVS7-151_152delGA, IVS7-99C→A, IVS8-35G→A, IVS8+28C→T, IVS10-18C→T, IVS11+21G→A, IVS12+15C→T, *207G→C, IVS12+32C→A) were found only in the XLMR panel but should be considered as unclassified variants or as a polymorphism (p.M560V). Our data indicate that the frequency of *SLC6A8* mutations in the XLMR population is close to that of CGG expansions in *FMR1*, the gene responsible for fragile-X syndrome.

Creatine and phosphocreatine play essential roles in the storage and transmission of phosphate-bound energy (Walker 1979; Wyss and Kaddurah-Daouk 2000). Humans maintain their creatine pool by creatine biosynthesis, which involves two enzymes—L-arginine:glycine amidinotransferase (AGAT; Enzyme Commission [EC] number 2.1.4.1) and guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2)—as well as nutritional uptake. Cellular transport is of fundamental importance for creatine homeostasis in tissues void of creatine biosynthesis. The creatine transporter gene (*SLC6A8/CT1/CRTR1* [MIM 300036]) has been mapped to Xq28 (Gregor et al. 1995) and is a member of the solute-carrier family 6 (neurotransmitter transporters). The *SLC6A8* gene spans ~8.4 kb; it consists of 13 exons (GenBank accession number Z66539) and encodes a protein of 635 amino acids with a predicted molecular weight of 70 kDa (Sandoval et al. 1996) (fig. 1).

The use of proton magnetic resonance spectroscopy (H-MRS) resulted in the identification of three inborn errors of metabolism: two creatine biosynthesis errors (AGAT deficiency [MIM 602360] and GAMT deficiency

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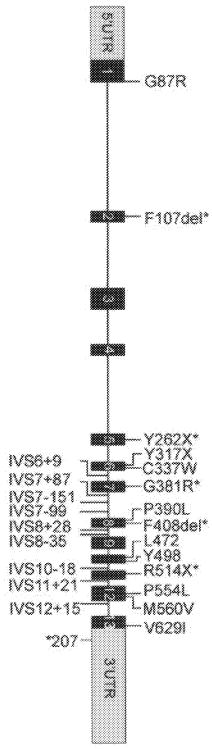


Figure 1 Mutations and polymorphisms in patients with XLMR. Primer sequences were designed specifically to amplify all exons of the *SLC6A8* gene (and not the *SLC6A10* gene, a presumed creatine transporter pseudogene mapped at chromosome 16 [Eichler et al. 1996]), including short fragments of the flanking intronic sequences. By comparing the *SLC6A8* and *SLC6A10* sequences, we have found that at least two nucleotide variations were present in all amplicons, confirming selective amplification of the *SLC6A8* sequences (data not shown). PCR reactions were performed with HotStar *Taq* (Qiagen) in a PE Applied Biosystems model 9700. Direct sequence analysis was performed on purified PCR products (Millipore vacufold) by use of BigDye v3.1 terminators and an ABI 3100 sequence machine (PE Applied Biosystems). The obtained electropherograms were assembled and analyzed to identify potential genomic alterations by use of the Mutation Surveyor software package (SoftGenetics). Sequence variants were annotated according the guidelines of den Dunnen and Antonarakis (2001). On the basis of impaired uptake in fibroblasts, five alterations (*asterisks [*]*) have been proven elsewhere to be mutations. p.F107del was also found in our XLMR cohort. One novel nonsense mutation (p.Y317X) is strongly predictive of impaired creatine uptake. p.M560V is a rare polymorphism, and p.V629I is an unclassified variant. The implications of the translational silent variant, the IVS variant, and the 3′ UTR variant could not yet be investigated, and these variants are therefore assigned as "unclassified." IVS variants in introns 1, 2, 3, and 4 may have been missed, since only small exon-flanking parts were included.

[MIM 601240]) and one creatine transport error (SLC6A8 deficiency [MIM 300036]) (Stockler et al. 1994, 1996*a*, 1996*b*; Schulze et al. 1997; Bianchi et al. 2000; van der Knaap et al. 2000; Cecil et al. 2001; Item et al. 2001; Salomons et al. 2001; Bizzi et al. 2002; deGrauw et al. 2002; Hahn et al. 2002; Salomons et al. 2003). These creatine-deficiency syndromes (CDS) share the almost-complete lack of creatine/phosphocreatine in the brain, as measured by in vivo H-MRS. Metabolite measurements in urine and plasma are indicative of the specific disorders (e.g., in SLC6A8-deficient males, the creatine:creatinine [Cr:Crn] ratio is increased in urine, and guanidinoacetate is increased in urine and plasma of GAMT-deficient patients). Characteristic in the clinical presentation of all CDS are mental retardation, expressive speech and language delay, and epilepsy (varying from intractable seizures in GAMT-deficient patients to mild epileptic or febrile seizures in AGAT-deficient and transporter-deficient patients). GAMT-deficient patients and transporter-deficient patients may show autistic behavior; in GAMT-deficient patients with a severe phenotype, (extra)pyramidal symptoms are present (Salomons et al. 2003; Stromberger et al. 2003). Female carriers of *SLC6A8* mutations may have learning disabilities and/or behavioral problems.

In Western countries, mental retardation (MR) affects 2%–3% of the general population (Chelly and Mandel 2001). For the majority of the cases of inherited MR, the genetic cause has not yet been elucidated. The larger number of males than females in the MR population (~70%) suggests a high contribution of X-linked disorders. X-linked mental retardation (XLMR) is estimated to account for 5%–12% of all cases of MR, with a relatively large (2%) contribution of fragile-X syndrome (fraX) (Herbst and Miller 1980; de Vries et al. 1997). The fact that >15 SLC6A8-deficient families (five families in one metropolitan area) have been identified in the 2 years after the first recognition of this disorder suggests a high incidence of creatine transporter deficiency in the Western population. Two prominent features of SLC6A8 deficiency are MR and X-linked inheritance of the disease. We therefore investigated by DNA sequence analysis the prevalence of *SLC6A8* defects in a panel of 290 unrelated families of the European XLMR Consortium (European XLMR Consortium Web site). Families are included in this panel if at least two males in the family are affected. Informed consent has been obtained from the parents or caretakers of the affected males. Of the 290 cases, 2 were excluded from this study, since we were unable to amplify, by PCR, DNA sequences of the *SLC6A8* gene and of two autosomal genes, because of the poor quality of the DNA. In addition, DNAs of 276 healthy controls were similarly analyzed.

In the European XLMR panel, seven novel nucleotide

substitutions in the ORF of *SLC6A8* were encountered: six missense variations/mutations and one insertion resulting in an immediate nonsense codon. In addition, one known pathogenic single–amino acid deletion was found. Furthermore, one translational silent change, eight intervening sequence (IVS) variants, and one substitution in the 3′ UTR were detected—none of which were found in either the 276 controls or the human EST database (table 1; fig. 1).

In DNA of the index patient of family T115, a deletion of 3 bp (c.319_321delCTT) in *SLC6A8* was detected, which results in the loss of phenylalanine at position p.107 (p.F107del). The mutation is located in a short repeat of two phenylalanines in exon 2. This repeat is part of transmembrane-spanning domain (TM) II, which is highly conserved among all known creatine transporters (i.e., bovine, rat, rabbit, marbled electric ray, and mouse [see fig. 2]) and within the Na^+- and Cl^--dependent neurotransmitter transporter family. The mutation segregates within the family (fig. 3*A*). *SLC6A8* F107del was previously reported in an unrelated family (de-Grauw et al. 2002) (fig. 1). The index male of that family showed impairment of creatine uptake in fibroblasts. The following observations indicate that p.F107del is pathogenic: (1) p.F107 is highly conserved between species; (2) p.F107 is located in a conserved area within its solute carrier family; (3) F107del segregates with the XLMR phenotype; (4) the mutation was detected in an unrelated family with SLC6A8 deficiency; (5) urine of affected males with this mutation showed an increased Cr:Crn ratio, a hallmark of this disease; and (6) fibroblasts with this deletion are impaired in creatine uptake.

In DNA of the index patient of family P66, an insertion of adenosine at position c.951 in exon 6 (c.950_951insA) of the *SLC6A8* gene was found. This mutation predicts a premature stop (p.Y317X) that results in a truncated protein of 316 amino acids that lacks 319 amino acids of the C-terminus, including the putative TM VII–XII of SLC6A8 (fig. 2). The mutation most likely results in an unstable and/or inappropriately folded protein that is completely inactive. Indeed, two other nonsense mutations in the *SLC6A8* gene (p.R514X and p.Y262X) (fig. 1) have been shown to impair creatine uptake and are pathogenic.

In DNA of the index patient of family T31, the transition G→A at position c.259 in the *SLC6A8* gene was found. This transition results in the substitution p.G87R. The mutation is located in a short repeat of three glycines in exon 1. This repeat is located between the putative TM I and II in a small intracellular loop that is highly conserved among all known creatine transporters and within the neurotransmitter transporter family SLC6 (fig. 2). The mutation results in the substitution of the nonpolar and neutral glycine for the polar and basic arginine. The highly conserved nature of p.G87 and the clear difference in the chemical properties of the wild-type and mutant amino acids indicate that the p.G87R mutation is pathogenic. Because no additional biological

Table 1

Nucleotide Changes in *SLC6A8* in the XLMR Panel

Genomic Mutation	cDNA Mutation	Deduced Effect	Family
g.2933G→A	c.259G→A	p.G87R[a]	T31
g.4533_4535delCTT	c.319_321delCTT	p.F107del[a]	T115
g.7400_7401insA	c.950_951insA	p.Y317stop[a]	P66
g.7461C→G	c.1011C→G	p.C337W[a]	N87
g.8032C→T	c.1169C→T	p.P390L[a]	T132
g.8883C→T	c.1661C→T	p.P554L[a]	D11
g.8900A→G	c.1678A→G	p.M560V[b]	P18
g.9291G→A	c.1885G→A	p.V629I[c]	N67
g.8467G→A	c.1416G→A	p.L472[c]	⩾1 family
IVS6+9C→T	ND	ND	⩾1 family
IVS7-151_152delGA	ND	ND	⩾1 family
IVS7-99C→A	ND	ND	⩾1 family
IVS8+28C→T	ND	ND	⩾1 family
IVS8-35G→A	ND	ND	⩾1 family
IVS10-18C→T	ND	ND	⩾1 family
IVS11+21G→A	ND	ND	⩾1 family
IVS12+15C→T	ND	ND	⩾1 family
*207G→C	ND	ND	⩾1 family
IVS12+32C→A	ND	ND	⩾1 family

NOTE.—The *SLC6A8* nucleotide changes shown in this table were not found in the 276 controls or in the human EST database. ND = not determined.

[a] Pathogenic mutation.

[b] Polymorphism.

[c] Unclassified variant.

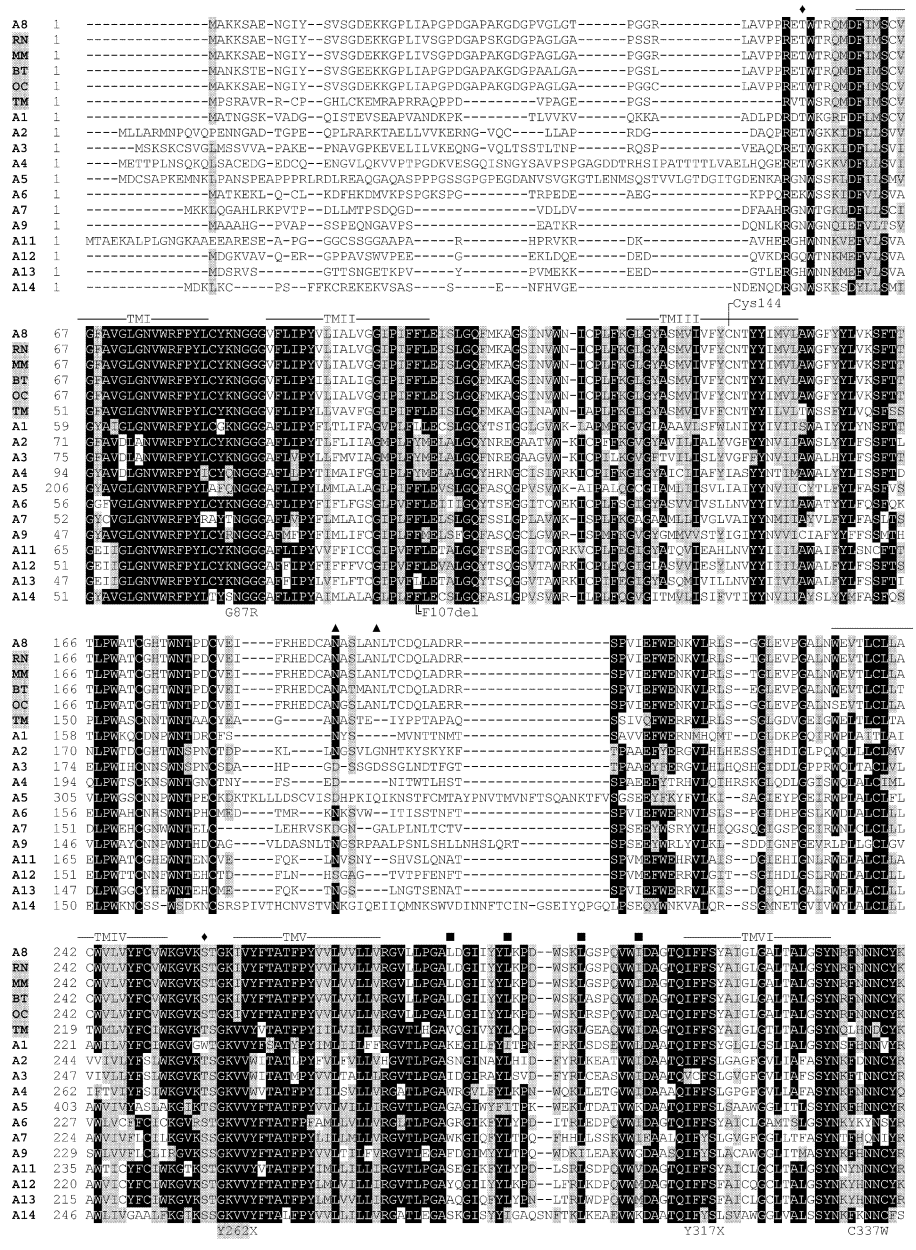
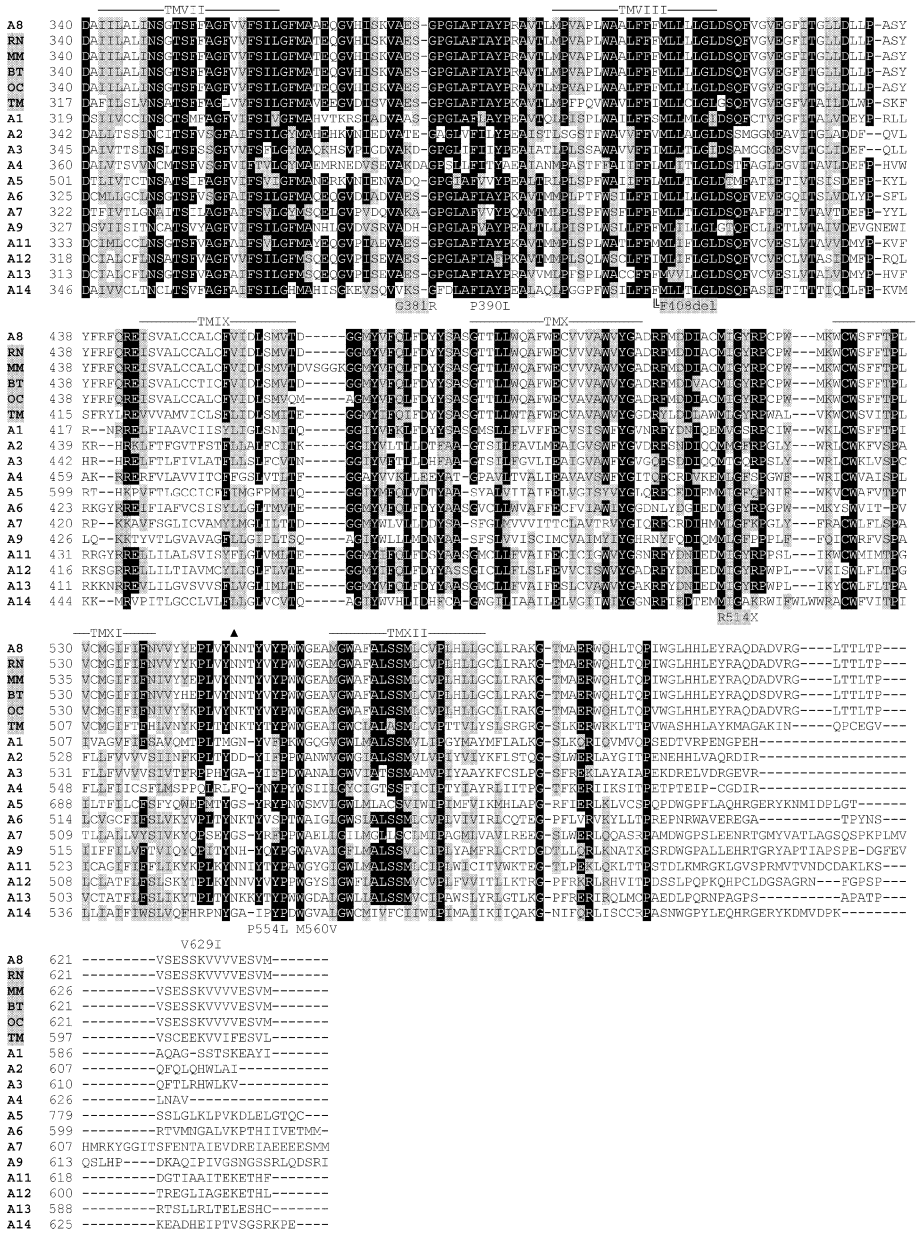


Figure 2 Multiple-sequence alignment with hierarchical clustering among the SLC6A8 proteins of different species and the superfamily of neurotransmitter transporters. The SLC6A8 protein sequence is shown for the following species: *Homo sapiens* (A8), *Rattus norvegicus* (RN), *Mus musculus* (MM), *Bos taurus* (BT), *Oryctolagus cuniculus* (OC), and *Torpedo marmorata* (TM). *SLC6A8* genes were identified by a protein blast (BLASTP). Alignment was determined by the ClustalW program; the SLC6A8 proteins that were used for this analysis were the ones identified by the BLASTP search to be most related to the *Homo sapiens* SLC6A8 protein. All were saved in FASTA format. The BOXSHADE program was used to visualize identical amino acids (*highlighted in black*) and functionally conserved amino acids (*gray*). Functionally conserved amino acids are classified as follows: V, I, L, and M; D, E, Q, and N; F, Y, and W; G, S, T, P, and A; and K, R, and H. The codes A1–A14 represent neurotransmitter transporters SLC6A1–SLC6A14 (A1 = GABA1, A2 = noradrenaline, A3 = dopamine, A4 = serotonine, A5 = glycine2, A6 = taurine, A7 = proline, A9 = glycine 1, A11 = GABA3, A12 = betaine/GABA, A13 = GABA2, and A14 = ATB^{0+}). In the protein sequence of A5, certain amino acids (20–46, 51–93, 116–127, 143–152, and 168–184) were cut out, because these stretches occur only in SLC6A5 and would make the figure disorganized. TMI–TMXII = putative transmembrane domain, predicted by ExPASy (Swiss-Prot S6A8_Human P48029); ♦ = putative cAMP-PK phosphorylation site (2 ×); ▲ = putative N-glycosylation sites (3 ×); ■ = putative Leu zipper motif (4 ×).

Reports



materials (e.g., fibroblasts or urine) were available, the pathogenicity could not be studied at the biochemical level or by H-MRS of the brain.

In two affected males of family N87, the transversion C→G at *SLC6A8* c.1011 was found, resulting in the substitution p.C337W. C337 is located in the intracellular loop between TM VI and VII and is highly conserved (fig. 2). The substitution of cysteine by tryptophan is highly significant: cysteine is polar and weakly acidic, and the sulfhydryl group is often involved in protein folding by forming disulfide links, whereas tryptophan is a nonpolar and neutral amino acid, with a very rigid cyclic structure. The patients' sister was a carrier of the p.C337W mutation (fig. 3*B*). In urine of the two affected males, an

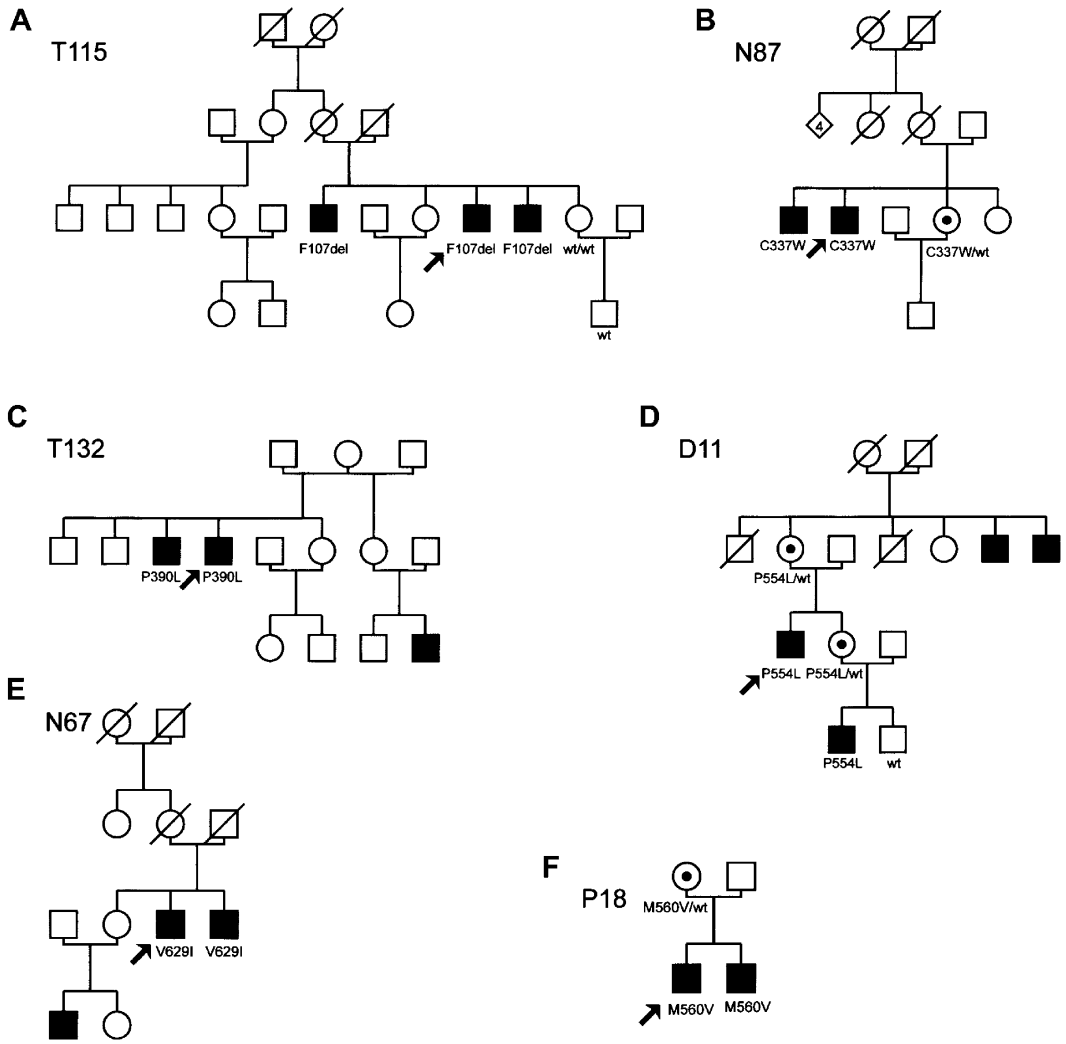


Figure 3 Pedigree charts of families in which segregation could be studied. (For designation of the variants, see table 1.)

increased Cr:Crn ratio was found, a biochemical hallmark of SLC6A8 deficiency. These data indicate that the p.C337W is pathogenic.

In DNA of the index patient of family T132, a transition of c.1169C→T was detected, resulting in the substitution p.P390L. The residue P390 is located in the extracellular loop between TM VII and VIII and is conserved in all known SLC6 family members, except for the serotonine transporter (fig. 2). The p.P390L substitution may have a significant effect on correct protein folding. An affected brother of the index patient also had the p.P390L substitution (fig. 3C). Therefore, we consider p.P390L a pathogenic mutation. It is unfortunate that, because of the unavailability of fibroblasts or urine, the pathogenicity could not be studied at the biochemical level or by H-MRS of the brain.

In DNA of the index patient of family D11, a transition of c.1661C→T was detected in exon 12 of *SLC6A8*, which resulted in the substitution p.P554L. P554 is a residue of the extracellular loop between TM XI and XII. The region

of TM IX–XI is expected to harbor the determinants of substrate specificity. The residue p.P554 is highly conserved (see fig. 2). The effect of a substitution of proline by leucine was discussed above. The p.P554L mutation segregates with the clinical phenotype within the family (fig. 3*D*). Thus, p.P554L is pathogenic. As discussed above, because of the lack of appropriate biological material from the affected males, the pathogenicity could not be studied at the biochemical or clinical level.

In DNA of the index patient of family N67, a G→A transition was detected at *SLC6A8* c.1885, which resulted in the substitution p.V629I in exon 13. The nonsynonymous substitution is located in a short repeat of four valines in the intracellular C-terminus of the creatine transporter protein (fig. 2). This residue is conserved in the creatine transporters between species but not among the SLC6 family members. Moreover, both valine and isoleucine are members of the same group of aliphatic amino acids, and, therefore, no change of chemical properties is predicted. The p.V629I mutation was also present in the affected brother (fig. 3*E*). Cr:Crn ratios in urine were within the low normal range (index patient and brother 0.001; control values 0–0.3). It is not known whether mutations in specific SLC6A8 regions could alter the function, stability, or trafficking of the protein, as is described for SLC6A2 (Bauman and Blakely 2002) and SLC6A13 (Brown et al. 2003). The c.1885G→A transition was not found in 276 controls and 95 ESTs. p.V629I is currently considered to be an unclassified variant.

In DNA of the index patient of family P18, an A→G transition was found in *SLC6A8* at c.1678 in exon 12, resulting in the substitution p.M560V. The substitution was also present in the affected brother of the index patient. The mother is a carrier of the transition (fig. 3*F*). p.M560 is conserved in rat, rabbit, and mouse creatine transporter but not in bovine (Val), marbled electric ray (Ile), or other members of the SLC6 family (fig. 2). The p.M560V mutation was not found in 276 control chromosomes. However, fibroblasts of the patient are not deficient in creatine uptake, and, in the H-MRS of the brain, the creatine signal was present. Therefore, we conclude that p.M560V is a rare polymorphism. Whether any of the above-mentioned silent, IVS, and 3′ UTR variants (table 1) are disease causing or just represent rare polymorphisms remains to be investigated.

The absence of missense mutations in 276 control chromosomes (appendix A) reduces the chance to <1% for a missense mutation to be a polymorphism with 80% power (Collins and Schwartz 2002). The size of the control population is rather small to provide conclusive information on the nature of a specific missense mutation found in the equal-sized XLMR population. On the other hand, the number of missense mutations differs significantly between the patient group and control groups (6/288 versus 0/276; Fisher's exact test P value = .03).

We have identified 6 pathogenic mutations in 288 patients. This means a prevalence of 2.1% SLC6A8-deficient patients in this XLMR panel. This may be an underestimate, since we were able to investigate only 93% of the coding sequence in 288 patients (the complete coding sequence in 180 patients and, overall, 80% of the coding sequence in the remaining 108 patients). In addition, some of the unclassified variants (n = 11) may prove to be pathogenic.

The presented results were calculated on the basis of our findings in a group of 288 patients who were preselected for suspected X-linked inheritance of MR. Currently, collaboration is being initiated to study the contribution of SLC6A8 deficiency in males with MR but without molecular defects in the *FMR1* gene (i.e., fraX negative).

Throughout exons 1–13 of the coding region of the *SLC6A8* gene, 14 previously reported and novel sequence variants have been identified. Mutation types include three nonsense mutations, two single–amino acid deletions in four families, one large deletion, four missense mutations, one combined missense/splice-site mutation, one unclassified variant (missense type), one translational silent mutation with unknown consequence, and one missense-type polymorphism. Although several mutations have been identified already, there is no indication of a recurrent mutation or a mutation-sensitive/-prone site (fig. 1).

Knockout-mouse models and clinical trials may result in a better understanding of the disorder and in appropriate treatment protocols for both affected males and females. The authors suggest that male patients with MR, autistic behavior, epilepsy, and/or expressive speech and language delay should be tested for creatine-deficiency disorders. Screening could be based on metabolite measurement of urine, H-MRS of the brain, and/or mutational analysis by direct sequencing of the gene(s). Functional tests could prove the biochemical defect and should include tests for AGAT and GAMT enzyme activity or creatine uptake into lymphoblasts or fibroblasts, respectively. SLC6A8 deficiency may contribute, together with fraX, to MR in an extensive proportion of males with nonsyndromic XLMR of unknown cause.

Acknowledgments

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Amsterdam, the University Medical Center in Nijmegen, and the University Medical Center in Leiden for providing the control DNA samples. This work was supported by ZonMw grant 940-37-031 and by European Union grant QLG3-CT-2002-01810 EURO-MRX.

Appendix A

Polymorphisms in the *SLC6A8* Gene

The anonymous DNAs of unaffected males were kindly provided by the Departments of Human Genetics of the VU University Medical Center in Amsterdam, the University Medical Center in Nijmegen, and the University Medical Center in Leiden.

SLC6A8 Polymorphisms in Controls and Patients with XLMR

IVS1+26G→A
IVS7+37G→A
IVS7+87A→G
IVS7-35G→A
IVS12-3C→T
c.1494C→T: p.Y498

SLC6A8 Polymorphisms in Controls Only

IVS2+88G→C
IVS9-36G→A
IVS12-82G→C

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/
European XLMR Consortium, http://www.euromrx.com/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC6A8* [accession number Z66539])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *SLC6A8,* AGAT deficiency, GAMT deficiency, and SLC6A8 deficiency)

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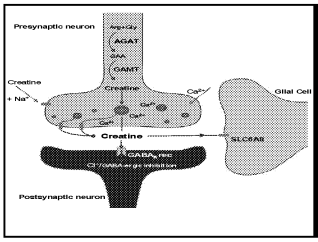
Presynaptic neuron
Arg+Gly
AGAT
GAA
GAMT
Creatine
Creatine
+ Na+
Ca2+
Glial Cell
Ca2+
Ca2+
Ca2+
Creatine
SLC6A8
GABAA rec
Cl-/GABA-evoked inhibition
Postsynaptic neuron

Chapter 6

Exocytotic Release of Creatine in Rat Brain

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KEY WORDS guanidinoacetate; neuromodulator; creatine; calcium; GABA

ABSTRACT The guanidino compound creatine has been shown to occur throughout the brain affecting energy metabolism and mental performance and to act at central $GABA_A$ receptors as a partial agonist. Therefore, we examined the possibility that creatine may in fact represent a neuromodulator that is released in the brain in an action-potential dependent manner. To that end, we studied the uptake of [^{3}H]creatine and its electrically evoked release from superfused rat brain slices as well as the evoked release of endogenously synthesized creatine. [^{3}H]creatine was accumulated in neocortex slices in a Na^+-dependent manner, consistent with the involvement of the Na^+-dependent SLC6A8 creatine transporter. Most importantly, the electrically evoked release of [^{3}H]creatine from neocortex slices (like that from caudate putamen and hippocampus slices) as well as the evoked release of endogenous (unlabeled) creatine was abolished when Ca^{2+} was omitted from the superfusion medium or in the presence of the Na^+-channel blocker tetrodotoxin (TTX). Moreover, blockade of K^+-channels by 4-aminopyridine (4-AP) strongly enhanced the electrically evoked release of [^{3}H]creatine as well as that of endogenous creatine. These in vitro data indicate that creatine is not only synthesized and taken up by central neurons, but also released in an action-potential dependent (exocytotic) manner, providing strong evidence for its role as a neuromodulator in the brain. **Synapse 60:118–123, 2006.**

INTRODUCTION

Creatine (α-*N*-methylguanidino acetic acid), synthesized from glycine, arginine and *S*-adenosylmethionine throughout the brain and in the periphery, is well known for its role in energy storage and transport/shuttle function of high-energy phosphates. Part of intracellular creatine is converted by creatine kinase into the high-energy compound, phosphocreatine. The creatine kinase isoforms are highly expressed in tissues with high and fluctuating energy demands, such as muscle and brain (Wyss and Kaddurah-Douk, 2000). Moreover, the creatine/phosphocreatine system is involved in neuronal growth cone activity and axonal elongation (Wang et al., 1998). Although the peripheral effects of creatine are well described, only a limited number of studies addressed its role in the brain. In this respect, the neuroprotective role of creatine has been most extensively studied (Klivenyi et al., 1999; Matthews et al., 1998, 1999; Tabrizi et al., 2003; Tachikawa et al., 2004). However, creatine does not only seem to be involved in energy metabolism, but may also play a modulatory role in a novel group of mental retardation syndromes denoted as cerebral creatine deficiency syndromes (Item et al., 2001; Salomons et al., 2001; Schulze et al. 1997; Stöckler-Ipsiroglu et al., 1994).

Considering the effects of creatine on central neurotransmission processes, it has been shown that guanidino compounds, including creatine, may affect GABAergic neurotransmission as (partial) agonists for $GABA_A$ receptors (De Deyn et al., 2001; De Deyn and Macdonald, 1990; Koga et al., 2005; Neu et al., 2002), suggesting a role of this compound as a central neuromodulator. To test this innovative hypothesis, we examined the uptake of [^{3}H]creatine in rat brain slices and the ionic characteristics of electrically evoked creatine release under quasi-physiological conditions in the present study.

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MATERIALS AND METHODS

Animals

Male Wistar rats (160–200 g body weight) were purchased from Harlan CPB, Horst (The Netherlands) and housed (two per cage) in a temperature- and humidity-controlled room with water and food available ad libitum. All experiments were approved by the Animal Care Committee of the Free University of Amsterdam.

Superfusion of brain slices

Determination of [^{3}H]creatine release

Rats were decapitated and slices of the neocortex, caudate putamen, or hippocampus (0.3 × 0.3 × 1 mm^3) were prepared using a McIlwain tissue chopper, then incubated with [^{3}H]creatine, and superfused essentially as described previously (Schoffelmeer and Mulder, 1983). In short, slices were washed twice with 5 ml Krebs–Ringer's bicarbonate medium containing 121 mM NaCl, 1.87 mM KCl, 1.17 mM $KHPO_4$, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$ (unless indicated), 10 mM D-(+)glucose, and pH 7.4. Subsequently, the slices were incubated for 15 min in this medium, containing 0.1 μM [^{3}H]creatine under an atmosphere of 95% O_2–5% CO_2 at 37°C. After labeling, the slices were washed and transferred to each of the 24 chambers of a superfusion apparatus (about 3 mg tissue per chamber, 0.2 ml volume) and superfused (0.25 ml/min), with medium gassed with 95% O_2–5% CO_2 at 37°C. EGTA (100 μM) was added to the superfusion medium when calcium was omitted in order to remove any remaining extracellular calcium in the slices. The superfusate was collected as 10 min samples after 40 min of superfusion (t = 40 min). [^{3}H]creatine release was induced during superfusion by exposing the slices to electrical biphasic blockpulses (electrical field-stimulation, 3 Hz, 24 mA, 2 ms pulses) for 10 min at t = 50 min. Drugs were added to the medium 20 min before stimulation of the slices.

Determination of endogenous creatine release

The superfusion procedure to measure the release of endogenously synthesized creatine was identical to that described earlier, except that the incubation step with [^{3}H]creatine was not performed. Endogenous creatine in superfusion fractions and tissue extracts was determined by stable isotope dilution gas chromatography–mass spectrometry (SID GC–MS) as previously described (Almeida et al., 2004). Briefly, the aqueous superfusion fractions were blown to dryness at 37°C using a gentle flow of nitrogen and resuspended in 100 μl water. To this 100 μl sample, 50 μl of saturated aqueous sodium bicarbonate, 50 μl hexafluoroacetylacetone, 500 μl toluene, and 50 μl internal standard (IS) solution in water (0.005 mM methyl-$^2H_3$-creatine) were added. The mixture was heated to 80°C for 2 h under continuous stirring and allowed to cool. From the upper toluene phase, 300 μl were transferred to another test tube and blown to dryness under nitrogen. Subsequently, 10 μl triethylamine and 100 μl 7% pentafluorobenzylbromide in acetonitrile (v/v) were added. Derivatization was carried out at room temperature for 15 min. Reaction was stopped by adding 200 μl 0.5 N HCl and the formed derivatives were extracted with 1 ml hexane. The hexane layer was finally transferred to a new vial and analyzed. Samples were injected in the GC–MS at a temperature of 300°C. Initial oven temperature was maintained at 100°C for 1 min followed by a ramp of 15°C/min to 260°C. The temperature of the transfer line was set at 300°C. The column (SGE BPX-70, Bester, Amstelveen, The Netherlands) was inserted directly into the ion source, which was set at 250°C. Quadrupole temperature was 150°C. Ammonia was used as moderating gas at an optimized gas pressure. The mass spectrometer (Hewlett Packard, type Engine) was operated under electron capture negative chemical ionization in the single ion-monitoring mode. The negative ions measured were m/z = −302 and −305 for creatine and IS, respectively.

Calculation of release data

To determine ([^{3}H])creatine release from superfused brain slices, the content of the tissue remaining at the end of the superfusion experiment was extracted from the tissue with 0.1 N HCl. The radioactivity in superfusion fractions and tissue extracts was determined by liquid scintillation counting. The efflux of radioactivity/unlabeled creatine during each collection period was expressed as a percentage of the tissue content at the beginning of the respective collection period. Electrically evoked ([^{3}H])creatine release was calculated by subtracting the spontaneous efflux from the total overflow during stimulation and the following 20 min. Because ([^{3}H])creatine release was returned to basal levels during the next 10 min period, a linear decline from the 10 min interval before to that 40–50 min after the onset of stimulation was assumed for calculation of spontaneous ([^{3}H])creatine. The evoked release was expressed as percentage of the content of the slices at the start of the stimulation period. Spontaneous [^{3}H]creatine release amounted to about 8% of total tissue tritium independent of the brain region studied. Spontaneous release of endogenous creatine in neocortex slices averaged 4.6% of total tissue creatine content.

The effects of drugs on evoked neurotransmitter release in excess of spontaneous efflux were analyzed using one-way ANOVA's, followed by Dunnett's multiple comparison test. In each experiment, quadruplicate observations were made.

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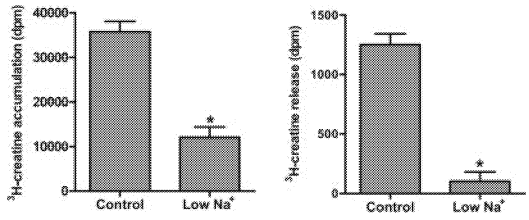

Fig. 1. Sodium-dependent uptake of [^{3}H]creatine in neocortex slices. Neocortex slices were incubated with [^{3}H]creatine in either normal Krebs–Ringer's bicarbonate medium or in medium in which NaCl was replaced by (121 mM) cholineCl. Subsequently, the slices were superfused and stimulated electrically for 10 min at t = 50 min. The amount of radioactivity (in dpm) present in the slices at the onset of electrical stimulation (an index for [^{3}H]creatine uptake) is represented in the left panel and the amount of radioactivity released in excess of spontaneous tritium efflux (an index of previously accumulated and releasable [^{3}H]creatine) in the right panel. Data represent means ± SEM of 12 observations. Per experiment quadruplicate observations were made. *Significantly different than that found in the presence of 121 mM NaCl ($P < 0.01$).

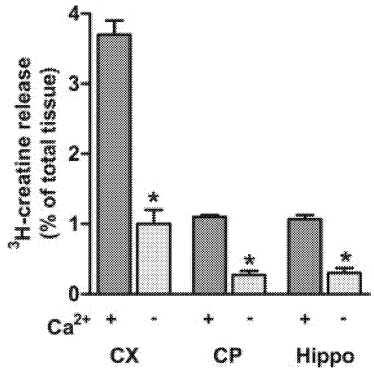

Fig. 2. Ca^{2+}-dependent electrically evoked [^{3}H]creatine release from superfused neocortex (CX), caudate putamen (CP), and hippocampus (Hippo) slices. Slices prelabeled with [^{3}H]creatine were superfused with medium containing either 1.2 mM Ca^{2+} or with Ca^{2+}-free EGTA (100 μM)-containing medium and stimulated electrically at t = 50 min for 10 min. The electrically evoked release of [^{3}H]creatine in excess of spontaneous tritium efflux is expressed as percentage of total tissue radioactivity in the slices present at the onset of electrical depolarization of neurons. Data represent means ± SEM of 12 observations. Per experiment quadruplicate observations were made. *Significantly different than that found in the presence of Ca^{2+} ($P < 0.01$).

Radiochemicals and drugs

[^{3}H]creatine (specific activity, 85 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Stable isotope-labeled creatine (methyl-$^2H_3$-creatine) was obtained from C/D/N isotopes (Quebec, Canada) and creatine, EGTA, 4-aminopyridine (4-AP), and tetrodotoxin (TTX) were from Sigma (St. Louis, MO).

RESULTS

Na^+-dependent [^{3}H]creatine uptake

The accumulation of [^{3}H]creatine by rat neocortex slices, as measured in superfused tissue following 50 min of superfusion (i.e., at the onset of electrical stimulation of the slices in subsequent experiments), amounted to about 70 pmol/mg tissue and was reduced by 70% when NaCl was replaced by cholineCl (though in the presence of 25 mM $NaHCO_3$) in the incubation as well as in the superfusion medium (Fig. 1). Moreover, electrical field-stimulation of the slices (3 Hz, 24 mA, and 2 ms pulses) during 10 min caused an increase of [^{3}H]creatine efflux. As shown in Figure 1, labeling of this readily releasable store also appeared to be Na^+-dependent, since the electrically evoked release (in excess of spontaneous efflux) was diminished upon lowering the Na^+ concentration in the incubation and superfusion buffer.

Action-potential dependent [^{3}H]creatine release

The electrically evoked [^{3}H]creatine release from superfused slices of the neocortex in excess of spontaneous tritium efflux amounted to almost 4% of total tissue radioactivity, while electrically evoked [^{3}H]creatine release was found to be more than 3 times lower when caudate putamen and hippocampus slices were used (Fig. 2). Figure 2 also indicates that, irrespective of the brain region studied, the electrically evoked [^{3}H]creatine release was strongly reduced when brain slices were superfused with Ca^{2+}-free EGTA (100 μM)-containing medium. Since Ca^{2+}-dependent [^{3}H]creatine release from neocortex slices was most prominent, we focused on the characteristics of electrically evoked neocortical release in our subsequent experiments.

As depicted in Figure 3, evoked [^{3}H]creatine release from neocortex slices was not only Ca^{2+}-dependent, but also dependent on transmembrane Na^+- and K^+-ion fluxes. Thus, blockade of voltage-gated Na^+-channels by 1 μM TTX strongly reduced evoked release, whereas blockade of K^+-channels by 100 μM 4-AP profoundly enhanced electrically stimulated [^{3}H]creatine release.

Release of endogenously synthesized creatine

In a final set of experiments, we examined the ionic requirements of electrically evoked release of creatine, synthesized rather than accumulated in neocortex slices. The electrically evoked creatine release in excess of spontaneous efflux from superfused slices (amounting to about 0.5% of total tissue creatine) was abolished in Ca^{2+}-free medium. Moreover, as shown regarding the release of [^{3}H]creatine, the evoked release of endogenous creatine was abolished by 1 μM TTX and potentiated by 100 μM 4-AP (Fig. 4).

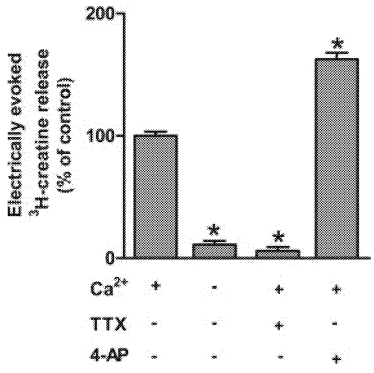

Fig. 3. Action-potential-dependent electrically evoked [^{3}H]creatine release from superfused neocortex slices. Slices prelabeled with [^{3}H]creatine were superfused with medium in the absence or presence of 1.2 mM Ca^{2+}, 1 μM TTX, or 100 μM 4-AP and stimulated electrically at t = 50 min for 10 min. Drugs (TTX, 4-AP) were added to the medium 20 min before electrical depolarization of neurons. The electrically evoked release of [^{3}H]creatine in excess of spontaneous tritium efflux is expressed as percentage of control release observed in the presence of Ca^{2+} and absence of drugs. Control release amounted to (3.65 ± 0.12)% of total tissue tritium. Data represent means ± SEM of 12 observations. Per experiment quadruplicate observations were made. *Significantly different from control release ($P < 0.01$).

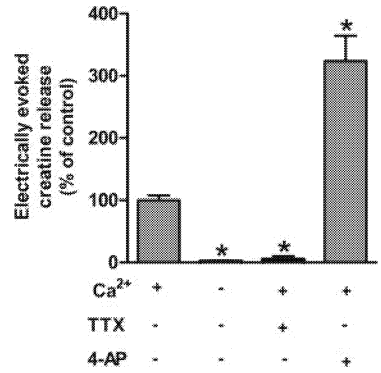

Fig. 4. Action-potential-dependent electrically evoked release of endogenous creatine from superfused neocortex slices. The slices superfused with medium in the absence or presence of 1.2 mM Ca^{2+}, 1 μM TTX, or 100 μM 4-AP and stimulated electrically at t = 50 min for 10 min. Drugs (TTX, 4-AP) were added to the medium 20 min before electrical depolarization of neurons. The electrically evoked release of creatine in excess of spontaneous tritium efflux is expressed as percentage of control release observed in the presence of Ca^{2+} and absence of drugs. Control release amounted to (0.49 ± 0.03)% of total tissue creatine. Data represent means ± SEM of 12 observations. Per experiment quadruplicate observations were made. *Significantly different from control release ($P < 0.01$).

DISCUSSION

Our present study is the first to show that the guanidino compound creatine, synthesized in neurons throughout the brain, is released from central neurons in a manner similar to that of classical neurotransmitters, i.e., involving an exocytotic release mechanism.

Previous studies indicated that creatine displays a neuroprotective effect in the brain as revealed in animal models for amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, ischemia, and trauma (Klivenyi et al., 1999; Matthews et al., 1998, 1999; Tabrizi et al., 2003). This guanidino compound seems to be essential for brain function, since others and we demonstrated that patients suffering from a cerebral creatine deficiency syndrome may have mental retardation, speech and language disorders, autistic features, extrapyramidal movement disorders, and epileptic seizures (Item et al., 2001; Salomons et al., 2001; Schulze et al., 1997; Stöckler-Ipsiroglu et al., 1994). In addition, guanidino compounds such as creatine are neurotoxic, presumably by altering the functioning of inhibitory and excitatory aminoacid receptors, underlying the epileptic and cognitive symptomatologies of uremic encephalopathy (De Deyn et al., 2001).

Considering its acute pharmacological effect on synaptic transmission, an early study showed that creatine reduces inhibitory GABA and glycine responses in cultured central neurons (De Deyn and MacDonald, 1990). In addition, Neu et al. (2002) showed that pathophysiological concentrations of guanidino compounds may cause neuronal hyperpolarization by enhancing the chloride conductance of $GABA_A$ receptors because of their action as partial agonists at these receptors. Moreover, in a recent study on stress-induced anxiety, creatine appeared to mediate an anxiolytic (sedative-hypnotic) effect as partial agonist at (the benzodiazepine binding site of) central $GABA_A$ receptors (Koga et al., 2005). In view of this partial agonist activity at $GABA_A$ receptors, it is conceivable that creatine modulates GABAergic neurotransmission throughout the brain, thereby acting either as an agonist or as an antagonist at $GABA_A$ receptors, depending on the local GABA concentration at these receptors. These acute receptor-mediated actions of creatine and the ubiquitous presence of this compound as well as its biosynthesis enzymes in central neurons (Braissant et al., 2005) suggest a putative role of creatine as a neuromodulator in the brain. In this respect, creatine might for instance be released as a cotransmitter (of e.g., GABA) upon arrival of an action potential at neuronal varicosities, modulating postsynaptic neurotransmitter (e.g. $GABA_A$) receptor functioning. This possibility is of particular interest because GABA represents the most widely occurring inhibitory neurotransmitter in the central nervous system. Thus, depending on the brain region, GABA is an inhibitory neurotransmitter in 20–50% of all central synapses, thereby displaying a higher affinity for (ionotropic) $GABA_A$ receptors than (metabotropic)

$GABA_B$ receptors (Sieghart, 1995). Of course, before accepting this idea, it is crucial to show that this guanidino compound is not only taken up and synthesized in central neurons, but also released in an action-potential dependent fashion, i.e., that the creatine is released upon depolarization of brain tissue in a Ca^{2+}-dependent manner involving activation of voltage-dependent Na^+- and K^+-channels (Orrego, 1979).

In agreement with such a putative neuromodulator role in the brain, creatine has been shown to be taken up in cells through the so-called SLC6A8 transporter that belongs to the well-known superfamily of sodium-dependent SLC6 neurotransmitter transporters for classical neurotransmitters such as monoamines, glycine, and GABA (Nash et al., 1994; Sora et al., 1994). Indeed, in our experiments, rat brain (neocortex) slices accumulated [^{3}H]creatine in a Na^+-dependent manner. As might then be expected, the absolute amount of [^{3}H]creatine release from superfused neocortex slices upon electrical field stimulation was diminished when preincubation of the tissue with [^{3}H]creatine was performed in medium in which NaCl was replaced by cholineCl. These initial results prompted us to further investigate the ionic requirements of the electrically evoked release of [^{3}H]creatine from superfused brain slices under experimental conditions identical to those used in numerous previous studies in brain slices (e.g., Middlemiss and Hutson, 1990; Orrego, 1979; Schoffelmeer et al., 1981, 2002). Interestingly, these experiments revealed that the electrically evoked release of [^{3}H]creatine was diminished in the absence of extracellular Ca^{2+} independent of the brain region examined (neocortex, caudate putamen, and hippocampus), indicating the involvement of an exocytotic release mechanism. Indeed, the occurrence of such vesicular [^{3}H]creatine release from depolarized central neurons was evident in our subsequent experiments in which pharmacological blockade of action-potential propagation by the highly selective Na^+-channel inhibitor TTX (1 μM) appeared to virtually abolish the electrically evoked release of [^{3}H]creatine from neocortex slices, as previously demonstrated regarding the in vitro release of radiolabeled classical neurotransmitters (see e.g., Limberger et al., 1986; Schoffelmeer et al., 1981). Moreover, depolarization-induced calcium-dependent [^{3}H]creatine release not only appeared to depend on the activation of voltage-gated Na^+-channels but also on that of K^+-channels. Accordingly, as shown regarding the release of e.g., noradrenaline and GABA from brain slices (Gu et al., 2004; Schoffelmeer and Mulder, 1983), blockade of voltage-sensitive K^+-channels by 100 μM 4-AP (Aronson, 1992; Smith et al., 2000) strongly increased its electrically evoked release.

As in most in vitro studies on the presynaptic regulation of central neurotransmitter release, the present experiments on depolarization-induced creatine release involved radioactive labeling of the putative releasable neurotransmitter store via the high affinity (SLC6) transporter for the compound. Therefore, in this case also it was of importance to address the physiological relevance of the results by investigating the electrically evoked release of endogenously synthesized creatine and its dependence on the ion permeabilities of nerve terminal membranes. This final set of experiments revealed an even more striking Ca^{2+}-, Na^+-, and K^+-ion dependence of the electrically evoked release of endogenous creatine than observed studying the release of the radioactive guanidino compound from superfused neocortex slices. Thus, no electrically evoked release of endogenously synthesized creatine was detectable when Ca^{2+} was omitted from the superfusion medium or when TTX was added to the medium, whereas blockade of K^+-channels by 4-AP caused a more than threefold increase in depolarization-induced creatine release.

Taken together, our data show that (radiolabeled) creatine is taken up in rat brain slices in a sodium-dependent manner and is subsequently released upon electrical depolarization of the tissue in a Ca^{2+}-dependent and TTX- and 4-AP-sensitive (action potential dependent) manner, just like that of the electrically evoked release of endogenously synthesized creatine and neurotransmitters such as amino acids and monoamines. Therefore, there can be little doubt that creatine release from central neurons involves an action-potential dependent exocytotic secretory process. However, from which type(s) of central neurons this vesicular creatine release originates remains to be investigated. In view of our data and available literature, we tentatively suggest that creatine represents a cotransmitter in the brain modulating the functioning of postsynaptic receptors for neurotransmitters such as GABA.

ACKNOWLEDGMENTS

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Arginine
Glycine
Urea Cycle
AGAT
Ornithine
Guanidinoacetic acid
Creatine
GLOBAS
Creatine
GAMT
SAM
SAH
Phosphocreatine
CK
ADP, ATP
Creatine
H2C
Creatinine
Hepatocytes
Creatine
Creatine
Creatine
Gut cell
Erythrocytes
Polymorphic nuclei

Chapter 7

Are cerebral creatine deficiency syndromes on the radar screen?

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Cerebral creatine deficiency syndromes (CCDS) are responsible for a considerable proportion of the population affected with mental retardation. CCDS are caused by either an inborn error of the proteins involved in creatine biosynthesis or in the creatine transporter. Besides mental retardation, the clinical characteristics of CCDS are speech and language delay, epilepsy and features of autism. CCDS can be diagnosed by proton magnetic resonance spectroscopy of the brain and/or by biochemical and molecular analysis. Treatment of the defects in creatine biosynthesis has yielded favorable outcomes, while treatments for creatine transporter deficiency are still under investigation at this time. The relatively large contribution of the CCDS to the monogenic causes of mental retardation emphasizes the importance of including CCDS in the differential diagnosis of mental retardation of unknown etiology. Pathophysiology is not yet unravelled, although it is known that creatine plays an important role in energy storage and transmission. Moreover, *in vitro* data indicate that creatine acts as a neuromodulator in the brain.

In the last decade, a novel group of inborn errors of proteins involved in creatine biosynthesis and its transporter has been identified. The high prevalence of these defects within the mentally retarded population and the promising treatment possibilities argue for the inclusion of cerebral creatine deficiency syndromes (CCDS) in the differential diagnosis of mental retardation of unknown etiology. CCDS arise by mutations in either one of the autosomal genes *AGAT* and *GAMT*, which encode the two enzymes (arginine:glycine amidinotransferase and guanidinoacetate methyltransferase, respectively) [1,2] necessary for creatine biosynthesis, or by mutations in the X chromosomal creatine transporter gene (solute carrier family 6 member 8 [*SLC6A8*]) [3]. Until recently, this group of syndromes has been termed creatine deficiency syndromes (CDS). However, in bodily fluids, no creatine deficiency exists in creatine transporter-deficient patients, thus, this term may be misleading. We therefore prefer to use the term CCDS, which also correlates better to the main clinical hallmarks that are related to CNS involvement.

In the general population, the incidence of mental retardation is estimated to be approximately 1% (profound, severe and moderate cases) to 3% if mild cases are included (IQ 50–70). Within the mentally retarded population, the frequency of X-linked mental retardation (XLMR) is estimated at 5–12% [4]. This number, and the fact that a large group of XLMR genes are already known [5], indicates that each monogenic cause of XLMR only accounts for a small percentage of mental retardation in males [6,7]. For the fragile X mental retardation gene (*FMR1*), the Aristaless-related homeobox gene (*ARX*) and, to a lesser extent, for the creatine transporter gene (*SLC6A8*), this seems to be different, as they account for a larger proportion of the XLMR subgroup [7].

The prevalence of SLC6A8 deficiency has been studied in three different patient groups. In the first panel, consisting of males with a strong predilection to having XLMR, a prevalence of 2.1% (six out of 288) was found. Considering the estimation that only 10% of the patients with mental retardation are affected with an X-linked defect, the prevalence of 2.1% in XLMR would translate to approximately 0.2% in the general mental retardation population of unknown etiology [8]. However, the prevalence in the two panels with mental retardation (four out of 478 = 0.8%, [9]) and global development delay (two out of 92 = 2.2%, [10]) was somewhat higher than predicted. This latter group was investigated by proton magnetic resonance spectroscopy (MRS), whereas the other groups were studied by DNA sequence analysis.

The prevalence of AGAT and GAMT deficiencies are not expected to be high, since they are autosomal recessive disorders. Indeed, only 29 GAMT- and five AGAT-deficient patients have been reported [11–15]. However, awareness of GAMT deficiency may be of utmost importance in Mediterranean countries, because a high carrier rate of a pathogenic *GAMT* mutation exists [16,17], and most of the GAMT-deficient patients are from this region.

Keywords: AGAT, cerebral creatine deficiency syndromes, creatine, GAMT, *GATM*, guanidino compounds, mental retardation, metabolic disorders, neuromodulator, SLC6A8, solute carrier family 6 member 8

future medicine

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The relatively high prevalence of CCDS highlights the importance of CCDS inclusion in the differential diagnosis of mental retardation of unknown etiology.

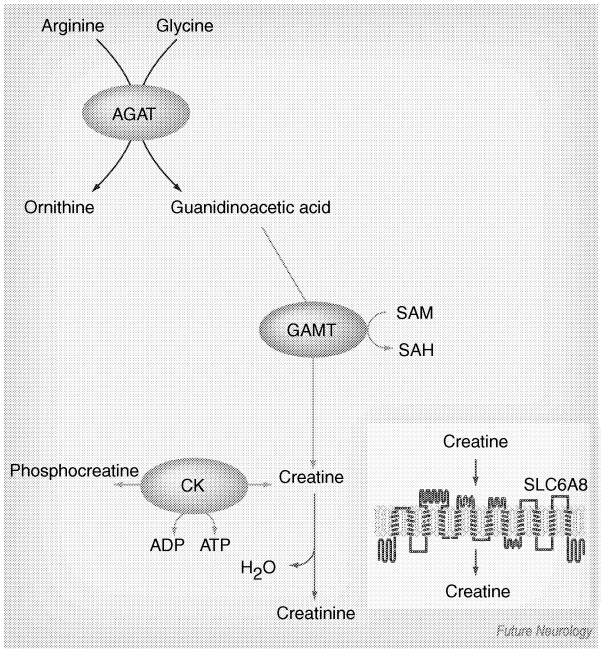
Inborn errors of proteins involved in creatine biosynthesis & its transporter

Arginine:glycine amidinotransferase deficiency

The initial and rate-limiting step in creatine biosynthesis is catalyzed by the AGAT enzyme, which forms guanidinoacetic acid, the precursor of creatine (**Figure 1**). In 2000, the first siblings with a defect in this enzyme were recognized [18]. These sisters (4- and 6-years old) presented with mental retardation and severe language delay. Routine blood and urine analyses were normal and further investigations did not suggest a neurometabolic disorder. Magnetic resonance imaging (MRI) of the brain was normal, but proton MRS revealed the absence of the creatine–phosphocreatine signal, suggesting a defect in the proteins involved in creatine biosynthesis or in the creatine transporter. In plasma, concentrations of creatine and guanidinoacetic acid were within normal values, which ruled out a deficiency of GAMT. The patients were reinvestigated in 2001, and analysis of urine revealed consistently decreased levels of guanidinoacetic acid [19]. The diagnosis of AGAT deficiency was confirmed by impaired AGAT activity in cultured cells and the detection of a pathogenic mutation in exon 3 of the *AGAT* gene [19].

Presently, five AGAT-deficient patients are known from two unrelated families, in whom reduced levels of GAA in body fluids have been detected [14,15]. The general clinical and biochemical findings are listed in **Table 1**.

Figure 1. Creatine biosynthesis.



Creatine biosynthesis involves a two-step reaction: the first is catalyzed by arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1) and the second by guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2). Creatine is transported via the bloodstream and taken up by tissues with high CK activity, such as muscle and brain, via a creatine transporter (SLC6A8). CK catalyzes the phosphorylation and dephosphorylation of creatine and phosphocreatine, respectively, thus providing a high-energy phosphate buffering system during ATP release and use.
ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CK: Creatine kinase.

Guanidinoacetate methyltransferase deficiency

The second step in creatine biosynthesis is mediated by the GAMT enzyme (**Figure 1**). In 1994, the first GAMT-deficient patient, a 22-month-old male was identified by Stöckler and collaborators [20,21]. The onset of symptoms started at 5 months with developmental arrest. The patient was hypotonic, unable to sit or roll over, showed uncoordinated swallowing and developed a severe extrapyramidal disorder. He had no organomegaly and his head circumference, hearing and vision appeared normal. Electrocardiogram and cardiac ultrasound examinations were normal; electroencephalogram showed low background activity and multifocal spikes. Nonspecific biochemical elevations were reported due to decreased creatinine levels. Magnetic resonance studies were performed for the first time at the age of 12 months: MRI revealed bilateral abnormalities in the globus pallidus. Proton MRS showed a spectrum lacking a creatine signal and an elevated guanidinoacetic acid peak. Treatment with arginine, the substrate of the AGAT enzyme, did not result in restoration of brain creatine. Moreover, guanidinoacetic acid in the brain, measured by proton MRS, remained increased, indicating that the defect was caused by a block in GAMT activity [20]. Indeed, in 1996, impaired GAMT activity in cultured cells and pathogenic mutations in the *GAMT* gene were identified [21]. Furthermore, a GAMT knockout mouse model has been developed, mimicking the biochemical characteristics of GAMT deficiency in humans [22–24].

Table 1. Overview of the patients described to date.

Cerebral creatine deficiency	*AGAT (n = 5)*[§]	*GAMT (n = 29)*[¶]	*SLC6A8 (n = 24)*[#]
Clinical traits:			
Age at diagnosis	0–5	0–26	2–66 years
Developmental delay	5/5	27/29	24/24
Speech and language delay	5/5	21/29	24/24
Mental retardation	5/5	27/29	24/24
Hypotonia	2/5	27/29	11/16
Behavior disorder	1/5	21/29	14/19
Movement disorder	NR	15/29	10/19
Seizures	NR[‡]	25/29	16/24
Mild phenotype*	NA	3/29	NA
Intermediate phenotype*	NA	12/29	NA
Severe phenotype*	NA	12/29	NA
H-MRS of the brain: absence/reduction Cr	5/5	27/29	12/12
Biochemical findings:			
Urinary creatine:creatinine			Increase in 17/17
Urinary guanidinoacetic acid	Decrease in 5/5	Increase in 29/29	Increase in 2/4
Plasmatic guanidinoacetic acid	Decrease in 5/5	Increase in 28/28	Increase in 1/2
Plasmatic creatine	Decrease in 5/5	Decrease in 28/28	NR
Treatment available	Yes	Yes	?

**Based on [11].*

[‡]Febrile seizures were reported in one of the first patients to be described [18].

[§] [14,15,19]; [¶] [11,12,13]; [#][9,53,66,67].

AGAT: Arginine–Glycine amidinotransferase; GAMT: Guanidinoacetate methyltransferase; NA: Not applicable; NR: Not reported.

To date, 29 patients have been described, varying from neonate to 29 years of age [11–13]. Owing to heterogeneous clinical presentation, GAMT-deficient patients can be classified as having a mild, moderate or severe phenotype, based on the severity grade of the main clinical characteristics (i.e., mental retardation, epilepsy and movement disorder). An overview of the clinical characteristics reported so far is presented in Table 1.

Creatine transporter deficiency

The creatine transporter, encoded by the *SLC6A8* gene, is essential for creatine uptake into cells. In 2001, the first patient with SLC6A8 deficiency was described. The patient presented with mild developmental delay at the age of 7 months in combination with central hypotonia. Prenatal and perinatal histories were unremarkable [25]. There was a history of learning disabilities and mental retardation in the family, compatible with an X-linked disorder. At 2 years of age the patient was admitted to hospital owing to a partial status epilepticus. Examination at 6 years of age showed a severe delay in speech and language development. A progressive increase of the head circumference (75th percentile to 95th percentile) prompted MRI and proton MRS. Proton MRS highlighted an almost complete loss of the creatine and phosphocreatine signal, similar to that observed in patients with AGAT and GAMT deficiency [26]. Consequently, oral creatine supplementation was commenced. However, after 4 months no restoration of cerebral creatine concentration was observed, which was in line with the lack of clinical improvement. Therefore, oral creatine supplementation was discontinued.

This, and the biochemical findings, ruled out a creatine biosynthesis defect. Moreover, high urinary creatine concentrations suggested a defect in cellular creatine transport. The inheritance pattern suggestive for X-linked disease and the fact that the gene encoding the creatine transporter, *SLC6A8*, is mapped to Xq28 [27], strengthened this hypothesis. This transporter is a member of the Na^+/Cl^--dependent neurotransmitter transporter family (Figure 2). Indeed, sequence analysis of *SLC6A8* revealed a hemizygous nonsense mutation. Furthermore, impaired creatine uptake in cultured fibroblasts was demonstrated [28]. The carrier status for this mutation was confirmed in the female relatives.

So far, more than 34 patients have been reported with SLC6A8 deficiency. The clinical characteristics of 24 male patients are listed in Table 1. In females, a very heterogeneous clinical phenotype is expected due to skewed X-inactivation, varying from learning disabilities to mental retardation.

How to diagnose cerebral creatine deficiency syndromes?

A marked reduction of cerebral creatine measured by proton MRS is highly indicative of a primary creatine deficiency syndrome. Workup by metabolite, molecular and functional studies is required to identify the underlying defect (i.e., AGAT, GAMT or SLC6A8 deficiency). Additionally, in families where individuals have previously been diagnosed with CCDS, prenatal diagnosis can be performed at the molecular level in chorion villus sampling or amniotic cells [12,15]. In case of GAMT deficiency, prenatal diagnosis can also be performed from the amniotic fluid at the metabolite level [12]. At present, the primary choice for

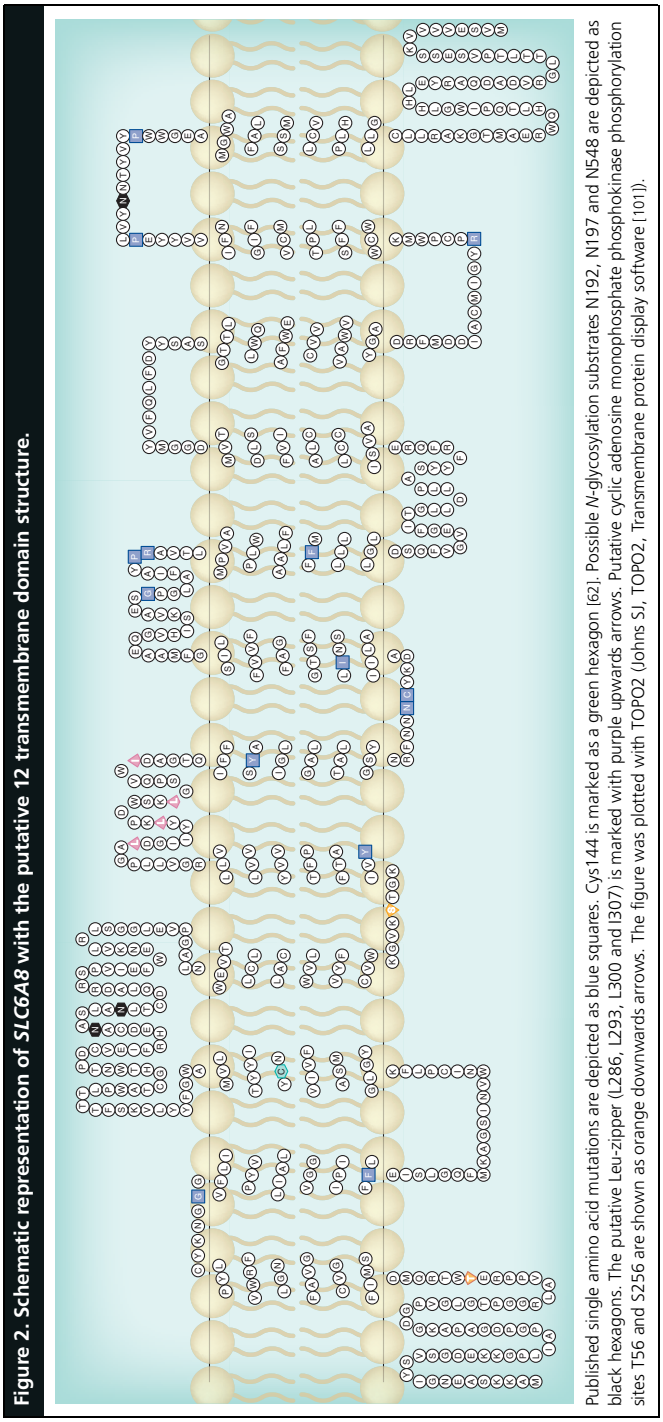


Figure 2. Schematic representation of *SLC6A8* with the putative 12 transmembrane domain structure.

Published single amino acid mutations are depicted as blue squares. Cys144 is marked as a green hexagon [62]. Possible *N*-glycosylation substrates N192, N197 and N548 are depicted as black hexagons. The putative Leu-zipper (L286, L293, L300 and I307) is marked with purple upwards arrows. Putative cyclic adenosine monophosphate phosphokinase phosphorylation sites T56 and S256 are shown as orange downwards arrows. The figure was plotted with TOPO2 (Johns SJ, TOPO2, Transmembrane protein display software [101]).

screening of CCDS in most institutes is measuring creatine, creatine:creatinine ratio and guanidinoacetic acid in body fluids or performing proton MRS of the brain. In contrast with metabolite analysis, proton MRS is expensive, not widely available and sedation is usually required.

Metabolite analysis

Various methods to measure creatine and guanidinoacetic acid in bodily fluids are available [29]. In diagnostic settings, the techniques generally used are gas chromatography-mass spectrometry [30,31], high-performance liquid chromatography (HPLC) [32] and tandem mass spectrometry [33–35]. Increased guanidinoacetic acid levels in bodily fluids are pathognomonic for GAMT deficiency, whereas reduced levels are found in AGAT deficiency. Creatine is usually reduced in the bodily fluids of patients with a biosynthesis defect, whereas an increased urinary creatine:creatinine ratio is found in males with SLC6A8 deficiency [30]. In the majority of females with a heterozygous *SLC6A8* mutation, this ratio is not informative.

Molecular diagnosis

Molecular diagnosis is usually performed by direct sequencing of the open reading frame of the respective gene or by mutation scanning using DHPLC [36] followed by DNA sequence analysis in case of aberrant heteroduplexes.

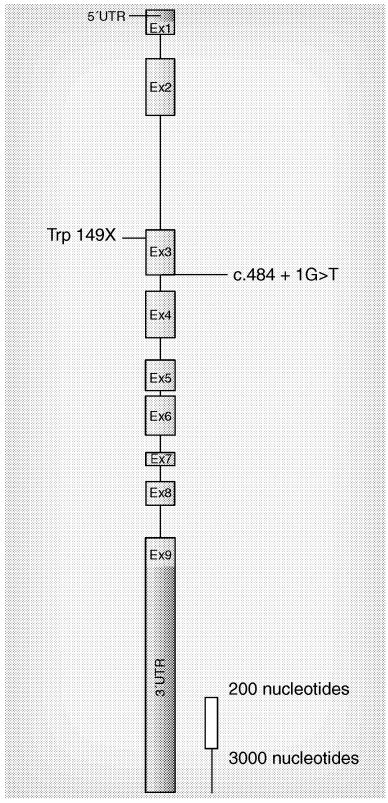
- The *AGAT* gene (Gene ID 2628, official nomenclature: *GATM*) has been mapped to chromosome 15q15.3, is 16.8 Kb in size and contains nine exons, which encode a protein of 424 amino acids. There are two mutations described (**Figure 3**);
- The *GAMT* gene (Gene ID 2593) has been mapped to chromosome 19p13.3, its size is 4.46 Kb and it contains six exons, which encode a protein of 237 amino acids. There are 15 pathogenic mutations described throughout the gene (**Figure 4**);
- The creatine transporter gene – *SLC6A8* (Gene ID 6535) has been mapped to Xq28 [37–39]. The *SLC6A8* gene spans 8.4 Kb consisting of 13 exons, which encode a protein of 635 amino acids. To date, 20 mutations have been described throughout *SLC6A8* (**Figure 5**).

Enzyme analysis

Several assays have been reported to assess both AGAT and GAMT activity [29]. The first methods to be reported used radioactive-labeled substrates [19,21,40,41]. In 2003, two methods using stable isotope labeled substrates were developed for both AGAT and GAMT [42,43]. The use of stable isotopes increases the sensitivity of the methods and reduces the amount of biological material required.

To study SLC6A8 deficiency, the creatine transporter function can be tested by a functional assay in fibroblasts [28].

Figure 3. Schematic representation of the *AGAT* gene and its reported mutations.

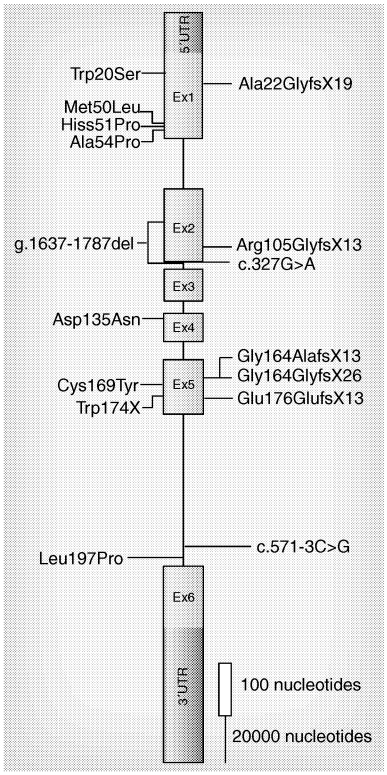


Exons (numbered boxes) and introns (black lines) are drawn to scale. To date, only two mutations have been reported (c.446G>A, p.Trp149X [19]; c.484 +1G>T, IVS3+1G>T [15]).

Diagnostic pitfalls

In the last decade, only two families with AGAT deficiency were reported, in comparison with 22 families affected with GAMT deficiency. AGAT-deficient patients could remain unnoticed

Figure 4. Schematic representation of the *GAMT* gene and its reported mutations.



Exons (numbered boxes) and introns (black lines) are drawn to scale. Splice-site and frameshift mutations are indicated on the right side; missense mutations are indicated on the left side; mutations are indicated in terms of protein. References for the mutations: c.59G > C, p.Trp20Ser; c.152A > C, p.His51Pro; c.160G > C, Ala54Pro; c.64dupG, p.Ala22fsX19; c.299_311dupGGGACTGGGCCCC, p.Arg105GlyfsX13; c.491delG, p.Gly164fsX13; c.521G > A, p.Trp174X; c.526dupG, p.Glu176GlufsX13; c.327G > A, IVS2+1G > A; g.1637_1787del [11]; c.506G > A, p.Cys169Tyr [16]; c.148A > C, p.Met50Leu [12]; c.491dupG, p.Gly164fsX26; c.571–3G > C, IVS5–3G > C [63]; c.590T > C, p.Leu197Pro [64].

in metabolic screening due to techniques that are neither sensitive or specific enough to detect decreased metabolite levels. Furthermore, intake of nutrition containing high concentrations of creatine (i.e., meat and fish) may hamper diagnosis of AGAT deficiency [44].

In approximately 10% of the males with mental retardation in whom elevated urinary creatine:creatinine ratio is found, no *SLC6A8* mutation is detected. They are likely to represent false-positive outcomes. Elevated urinary creatine:creatinine ratio may also be the secondary result of other (neuro)muscular disorders, such as Duchenne muscular dystrophy and Becker muscular dystrophy [45]. In rare cases, promoter mutations or deep intronic mutations may have been missed.

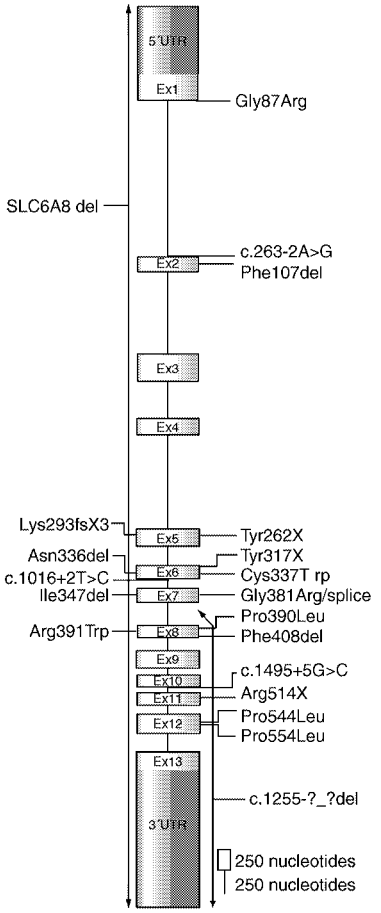
Although three female SLC6A8-deficient patients were found with an elevated urinary creatine:creatinine ratio, this is not the rule for the majority of heterozygous females [29]. Proton MRS of the brain in females may be restricted by difficulties in detecting minimal reductions in brain creatine and sedation is often required. Currently, in females, molecular screening is probably the most sensitive method for the detection of SLC6A8 deficiency, and thus urinary creatine:creatinine ratio is not the primary choice. In molecular diagnostic screening, care should be taken, since a paralogous gene, *SLC6A10*, which is mapped at chromosome 16p11, is highly identical to *SLC6A8* [46].

Regarding DNA sequence analysis, it should be noted that, in rare cases, unique sequence variations are detected, both in coding and noncoding regions in the *SLC6A8*, *GAMT* or *AGAT* gene, which were not encountered in controls [8,9,16]. Thus, it is important to evaluate the nature of the variants carefully, before classifying them as pathogenic mutations or polymorphisms.

Treatment of creatine biosynthesis defects & creatine transporter defects

Treatment approaches of CCDS aim to restore creatine in the brain by creatine supplementation, with success for the biosynthesis defects [20,47,48]. For AGAT deficiency, the highest success rate is expected because there is no accumulation of substrates. In fact, if started in a presymptomatic phase, creatine supplementation in AGAT and GAMT-deficient patients leads to an impressive restoration of cerebral creatine levels, and a favorable clinical response. In an AGAT-deficient infant, treatment initiated at 4 months of age showed normal development by 18 months of age, and the restoration of creatine levels in bodily fluids and the brain were almost complete [49]. Similar success was observed with a GAMT-deficient patient in whom presymptomatic treatment was initiated at 22 days of age [13]. This suggests that creatine supplementation in early life prevents the neurological sequelae.

Figure 5. Schematic representation of the *SLC6A8* gene and its reported mutations.



Exons (numbered boxes) and introns (black lines) are drawn to scale. References for the mutations: c.259G > A, p.Gly87Arg; c.319_321delCTT, p.Phe107del; c.950_951insA, p.Tyr317X; c.1011C > G, p.Cys337Trp; c.1169C > T, p.Pro390Leu; c.1661C > T, p.Pro554Leu [8]; c.263–2A > G, IVS2–2A > G [65]; c.319_321delCTT, p.Phe107del; c.786C > G, p.Tyr262X; c.1221_1223delCTT, p.Phe408del; c.1141G > C, p.Gly381Arg/splice; c.1540C > T, p.Arg514X [66]; c.878_879delTC, p.Lys293fsX3; c.1221_1223delCTT, p.Phe408del; [53]; c.1006_1008delAAC, p.Asn336del; c.1016+2T > C, IVS6+2T > C; c.1059_1061delCTT, p.Ile347del; c.1171C > T, p.Arg391Trp [9]; c.1255?_?del; *SLC6A8* del [67].

Currently, the most extensive experience has been obtained with the treatment of GAMT-deficient patients. The aim of the treatment is to increase the creatine and decrease the guanidinoacetic acid in brain. The severe phenotype observed in some cases in GAMT-deficient patients might be due to both the lack of creatine, but also due to guanidinoacetic acid accumulation, since guanidino compounds are known for their neurotoxic and epileptogenic effects [50]. In order to lower guanidinoacetic acid levels, AGAT activity needs to be reduced. This can be partly achieved by two different strategies:

- Substrate deprivation of AGAT by dietary restriction of arginine intake;
- With supplementation of high concentrations of ornithine and creatine.

With this therapeutic approach, levels of guanidinoacetic acid are lowered by 50% in bodily fluids [51]. This led to an additional clinical improvement as compared with treatment with creatine alone.

No successful treatment has currently been reported for SLC6A8 deficiency. Attempts with creatine supplementation in males showed no marked improvement [25,28,52,53]. Current trials are aiming at stimulation of creatine biosynthesis in the brain by supplementation with high doses of arginine and glycine, combined with high doses of creatine (Mancini GM, van der Knaap MS, Salomons GS, Unpublished Data). However, despite the expression of biosynthesis proteins in the brain, the spatial distribution of AGAT, GAMT and SLC6A8 may pose problems for this approach [54,55].

Creatine: a novel neuromodulator?

Whilst the function of creatine in energy metabolism has been addressed extensively, only a limited number of studies have focused on its role in the brain. Creatine synthesis has been observed in the CNS [56]. *In situ* hybridization studies also found *AGAT* and *GAMT* expression in almost all CNS cell types, in addition to its expression at the blood–brain barrier level, whereas SLC6A8 mRNA was only found in neurons, oligodendrocytes and brain capillary endothelial cells [54,55,57]. These data support the recent postulations that the brain is able to synthesize creatine.

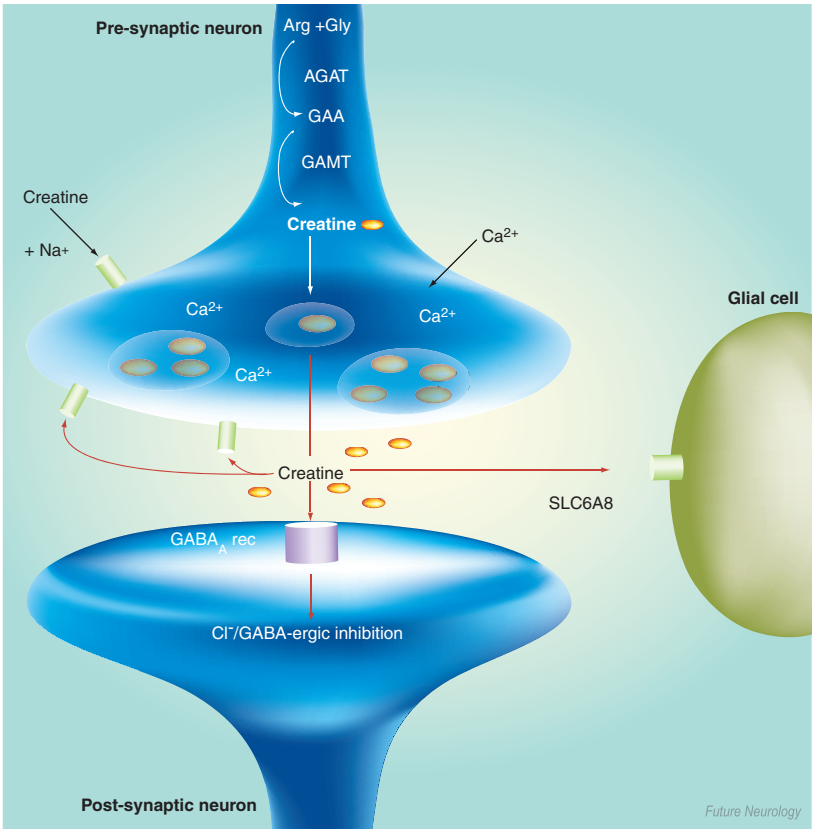
Creatine seems to be essential for brain function, as patients suffering from a CCDS have mental retardation, speech and language disorders, autistic features and may have extrapyramidal movement disorders and epileptic seizures (see above). Additionally, several guanidino

compounds are neurotoxic, presumably by altering the functioning of inhibitory and excitatory amino-acid receptors [58]. Considering the effects of creatine on central neurotransmission processes, it has been shown that guanidino compounds, including creatine, may affect γ-aminobutyric acid (GABA)-ergic neurotransmission as (partial) agonists for $GABA_A$ receptors [58–60]. Indeed, it was shown that creatine is released from central neurons in a manner similar to that of classical neurotransmitters, specifically, involving an exocytotic release mechanism [61]. In view of this *in vitro* data, it was hypothesized that creatine represents a cotransmitter in the brain that modulates the functioning of postsynaptic receptors for neurotransmitters, such as GABA (**Figure 6**) [61].

Conclusions

In the last decade, a novel group of inborn errors of metabolism has been identified: the cerebral creatine deficiency syndromes. The importance of considering CCDS in the differential diagnosis of mental retardation is emphasized by the relative high frequency of SLC6A8 deficiency, and the promising results of treatment of GAMT- and AGAT-deficient patients. The diagnostic tests to detect these syndromes are readily

Figure 6. Proposed model of action for cerebral creatine.



Creatine is synthesized in neurons, by the enzymes AGAT and GAMT. Upon synthesis creatine is released in a Ca^{2+} dependent (exocytotic/vesicular) manner into the synaptic cleft interacting with the $GABA_A$ receptors of the postsynaptic neuron. Creatine may subsequently be taken up from the cleft via the creatine transporter into the neurons or glial cells [61].
AGAT: Arginine:glycine amidinotransferase; GAA: guanidinoacetic acid; GABA: γ-amino butyric acid; GAMT: Guanidinoacetate methyltransferase.

available (e.g., metabolite and molecular analysis and, to a lesser extent, proton MRS), which makes this feasible for any institution that has access to at least one of these techniques. Moreover, proton MRS/MRI in particular enables the detection of multiple diseases. The recent findings that creatine acts as a neuromodulator opens up new research areas, which may be worthwhile for the elucidation of the pathophysiology of CCDS.

Future perspectives

Within the next 5–10 years in the Western world, every mentally retarded patient (male/female) will be screened for CCDS. This could be achieved primarily by proton MRS, which will certainly become more widely available, with the advantage of disclosing additional diseases. For the screening of CCDS, proton MRS is adequate, as a marked reduction or absence of the creatine signal is diagnostic for primary creatine deficiency. Currently, molecular analysis is performed by direct DNA sequencing. However, current developments in the field of microarrays allow the analysis of multiple genes, or even full genomes, in a single assay, which opens up a new world in both research and clinical applications. This technology may lead to more insights into the pathophysiology. The development of sequencing chips (i.e., resequencing arrays) also discloses new perspectives in the diagnosis and screening of CCDS, although its sensitivity is not yet acceptable for clinical diagnostics.

Treatment of SLC6A8 deficiency is one of the big challenges. Clinical improvement has been observed in the patients with biosynthesis defects upon treatment, with almost complete restoration of creatine in the brain. This proves that cerebral creatine restoration is essential. When a vehicle for creatine uptake in the brain is found, treatment should also be successful for SLC6A8 deficiency. Elucidation of the creatine biosynthesis pathway in the brain, in addition to the clarification of the function of creatine in the brain, may increase the success rate of treatment.

Tandem mass spectrometry has the potential for simultaneous multidisease screening and has recently been applied to neonatal screening programs. In case measurement of guanidinoacetic acid in dried blood spots proves to be specific and sensitive enough for detection/exclusion of GAMT deficiency, this disorder will be included in neonatal screening [32,33,13]. AGAT and SLC6A8 deficiencies are not yet eligible for neonatal screening since creatine and creatinine do not seem to be informative in the neonatal period.

Acknowledgements

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Executive summary

Inborn errors of proteins involved in creatine biosynthesis & its transporter

- Three novel disorders in creatine metabolism have been identified. Two autosomal recessive creatine biosynthesis defects (*AGAT* and *GAMT* deficiency) and one X-linked creatine transporter defect (*SLC6A8* deficiency). This group is defined as cerebral creatine deficiency syndromes (CCDS).
- The common hallmarks of CCDS are the absence, or marked reduction of the creatine signal in the proton magnetic resonance spectroscopy (MRS) of the brain, mental retardation and speech and language delay (Table 1).
- Worldwide, 29 patients with *GAMT* deficiency, five with *AGAT* deficiency and over 34 with *SLC6A8* deficiency have been reported.

High prevalence of cerebral creatine deficiency syndromes in the mental retardation population

- The prevalence of *SLC6A8* deficiency in patients with mental retardation is relatively high (~1%) compared with most monogenic causes of mental retardation.
- The prevalence of the autosomal recessive disorders AGAT- and GAMT-deficiency, is not high, however, a high carrier rate of a *GAMT* (founder) mutation exists in Mediterranean countries.
- The relatively high prevalence of CCDS highlights the importance of including them in the differential diagnosis of mental retardation of unknown etiology.

How to diagnose cerebral creatine deficiency syndromes?

- CCDS may be found either by proton MRS, metabolite screening and/or molecular investigations.
- Biochemically, AGAT-deficiency is characterized by low creatine and guanidinoacetic acid levels in bodily fluids, whereas GAMT-deficiency is characterized by elevated levels of guanidinoacetic acid in bodily fluids.
- Elevated urinary creatine:creatinine ratios are found in males affected with SLC6A8 deficiency.
- In females, the urinary creatine:creatinine ratio is not usually informative in this X-linked disorder, thus mutation analysis of *SLC6A8* is currently the primary choice.
- Prenatal diagnosis of CCDS is possible in families with affected individuals.

Executive summary

Diagnostic pitfalls

- AGAT deficiency is likely under-diagnosed because the biochemical tests may not be sensitive or specific enough to detect decreased metabolite levels.
- In approximately 10% of males with mental retardation in whom elevated urinary creatine:creatinine is found, no *SLC6A8* mutation is detected.
- False-negative biochemical findings (i.e., normal urinary creatine:creatinine ratio) are found frequently in females with heterozygous *SLC6A8* mutations.
- In molecular diagnostic screening, proper criteria should be used to classify novel sequence variants in the CCDS genes as pathogenic mutations.

Treatment of creatine biosynthesis defects & creatine transporter defect

- Treatment approaches of CCDS aim to restore cerebral creatine levels.
- The successful treatment of creatine biosynthesis defects consists primarily of daily creatine supplementation, which preferably should be initiated early in life. In addition, in GAMT deficiency, guanidinoacetic acid levels can be reduced by arginine restriction and ornithine supplementation.
- Current trials for the treatment of SLC6A8 deficiency aim at the stimulation of cerebral creatine biosynthesis by supplementation with high dosage of the substrates of the AGAT enzyme (i.e., arginine and glycine).

Creatine: a novel neuromodulator?

- Cerebral creatine biosynthesis has been proven by the demonstration of *AGAT* and *GAMT* mRNA expression in the brain.
- *In vitro* data indicate that creatine represents a novel cotransmitter in the brain.

Conclusions

- Cerebral creatine deficiency syndromes are, in part, treatable disorders.
- Diagnostic tests for the detection of cerebral creatine deficiency syndromes are widely available.
- Cerebral creatine deficiency syndromes should be included in the differential diagnosis of mental retardation, owing to their high frequency.

Future perspectives

- In the Western world, every mentally retarded patient (male/female) will be screened for CCDS, primarily by proton MRS.
- Novel techniques will increase the number of patients detected with CCDS.
- By identifying a vehicle for creatine uptake in the brain, treatment will also become available for patients with SLC6A8 deficiency.
- GAMT deficiency will be included in neonatal screening programs as guanidinoacetic acid seems to provide a suitable marker, as measured in blood spots by tandem mass spectroscopy.

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Website

101. Transmembrane protein display software. www.sacs.ucsf.edu/TOPO2

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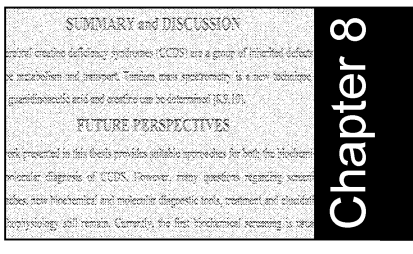
SUMMARY and DISCUSSION
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Chapter 8

SUMMARY and DISCUSSION

The cerebral creatine deficiency syndromes (CCDS) are a group of inherited defects in creatine metabolism and transport. Patients affected with CCDS are often diagnosed based on the levels of creatine and guanidinoacetic acid in body fluids (plasma, urine and CSF). We developed a method for the simultaneous analysis of creatine and guanidinoacetic acid, using stable isotope dilution gas chromatography-mass spectrometry (SID GC-MS). This method was used to establish reference values for creatine and guanidinoacetic acid in body fluids. These reference values were found to be age dependent; the concentrations of both compounds decrease with age. No differences between males and females were observed (**Chapter two**). So far, we have shown that patients affected with CCDS could be distinguished from controls in all cases. For the diagnosis of the X-linked creatine transporter defect, the creatine/creatinine ratio in urine is the only marker at the metabolite level. For the creatine biosynthesis defects, both plasma and urine samples can be used. We expect that for AGAT deficiency, plasma is the best choice, as decreased concentrations of guanidinoacetic acid and creatine are expected in these patients. In controls, a very low urinary concentration of guanidinoacetic acid can be found, and thus decreased levels - as expected in AGAT deficiency - may be hard to detect. The establishment of reference values for guanidinoacetic acid and creatine is of pivotal importance in the screening and detection of CCDS. Furthermore, this biochemical approach is easily applicable for the screening of CCDS for patients with unexplained mental retardation, dysphasia, epilepsy, and/or autistic behavior.

We proved that overexpression of GAMT could restore GAMT activity in deficient primary fibroblasts (**Chapter three**). Wild-type GAMT ORF was cloned into a pEGFP-N1 expression vector. This construct was introduced into deficient primary fibroblasts through stable transfections. Since fibroblasts are difficult to transfect, we also transiently transfected the construct into HeLa cells. Transfection of HeLa cells with wild-type GAMT resulted in a 56-fold increase of GAMT activity, compared to mock transfectants (cells transfected with the expression vector only). It may be arguable that the high levels of GAMT overexpression do not represent physiological conditions, however, this does not pose a problem in a diagnostic setting, in which the

main question is whether a mutation in the GAMT coding sequence will effect enzyme activity or not. In conclusion, we reported an approach to investigate the nature of variants in the *GAMT* gene.

The CCDS are a relatively new group of inborn errors of metabolism. Therefore, data on their prevalence is still scarce. It should be noted that GAMT deficiency was first reported 10 years ago [1], whereas AGAT deficiency and SLC6A8 deficiency were only described in 2001. The prevalence of AGAT and GAMT deficiencies, autosomal recessive disorders, is expected to be lower than the X-linked creatine transporter deficiency syndrome. Indeed, only 27 GAMT deficient and 5 AGAT deficient patients have been reported [2,3,4,5]. However, awareness of GAMT deficiency may be of utmost importance in Mediterranean countries. In **chapter four** we studied the carrier rate of a frequent mutation in the GAMT gene, c.59G>C; p.Trp20Ser. Using the approach developed in chapter three we investigated whether this mutation was indeed disease causing. From the 27 GAMT deficient patients reported to date, 18 are from Mediterranean countries and in 22 out of the 36 alleles they carry the same mutation, c.59G>C; p.Trp20Ser. From the 18 Mediterranean patients 10 are from Portugal carrying in 17 out of the 20 alleles this mutation. We obtained bloodspots from different regions of Portugal (Archipelagos, Porto, Coimbra, Lisboa, Faro) and screened the DNA's for the presence of the c.59G>C; p.Trp20Ser mutation by SNaPshot (Single Nucleotide Polymorphism Multiplex Kit). From the 1002 bloodspots analyzed, eight heterozygous subjects were detected suggesting a carrier rate of 0.8% (CI 0.2-1.3%). In a subgroup from the Archipelagos the carrier rate could be as high as 1.68% (4 heterozygotes out of 238 tested; CI 0-3.3%). Thus, our study suggests that the prevalence of the c.59G>C; Trp20Ser is relatively high in some regions of Portugal. We also proved that the c.59G>C; Trp20Ser missense mutation is pathogenic.

In **chapter five** we investigated the prevalence of the X-linked SLC6A8 deficiency. By DNA sequence analysis we studied the prevalence of SLC6A8 mutations in a panel of 290 patients with nonsyndromic X-linked mental retardation (panel provided by the European XLMR consortium). From the 288 studied patients six pathogenic mutations (four missense, one nonsense mutation and one aminoacid deletion) were identified suggesting a prevalence of 2.1%. Recently, two additional studies on prevalence of SLC6A8 deficiency have been reported. The prevalence in the two panels of patients with mental retardation and the second with global

developmental delay was 0,8% and 2% respectively [6,7]. Altogether these studies indicate that SLC6A8 deficiency is a frequent cause of mental retardation.

Creatine is mainly known for its function in muscle. Considerable interest in this compound has grown among the scientific community after the discovery of GAMT deficiency, the first inborn error of creatine metabolism reported. Creatine also occurs throughout the brain affecting energy metabolism and mental performance and was shown to act at central $GABA_A$ receptors as a partial agonist. The brain is the major organ affected in patients with CCDS. Therefore, in **chapter six,** we investigated if creatine represents a neuromodulator in brain. Using rat brain slices we studied the uptake of [^{3}H]-creatine and its electrically evoked release as well as the evoked release of endogenously synthesized creatine. Our *in vitro* data showed that creatine is not only synthesized and taken up by neurons but also released in an action-potential dependent exocytotic secretory process suggesting that creatine has a role as a neuromodulator in the brain.

In **chapter seven** we reviewed the latest developments in the CCDS field. The three disorders are reviewed focusing on clinical, biochemical and molecular aspects. An update from the patients reported so far is also provided.

In conclusion, considering CCDS in the differential diagnosis of mental retardation is of utmost importance as illustrated by the relative high frequency of SLC6A8 deficiency, and to a lesser extent of GAMT deficiency (specially in Mediterranean countries), and the possibility of treatment (more successful for the biosynthetic disorders). Diagnostic tests to screen and confirm these syndromes are readily available (e.g. metabolite and molecular analysis). Our recent findings that creatine acts as a neuromodulator opens new research areas which may lead to the elucidation of pathophysiology of CCDS.

FUTURE PERSPECTIVES

The work presented in this thesis provides suitable approaches for both the biochemical and molecular diagnosis of CCDS. However, many questions regarding screening approaches, new biochemical and molecular diagnostic tools, treatment and elucidation of pathophysiology still remain.

Currently, the first biochemical screening is usually performed by quantification of the biochemical markers (guanidinoacetic acid, creatine, creatine/creatinine ratio) in body fluids. In addition, proton magnetic resonance spectroscopy (MRS) of the brain provides an useful tool in the diagnosis of CCDS, as in all three defects the creatine signal is decreased or absent. At present, this technology is not available in all diagnostic centers, but its availability is expected to increase in the next years. Nonetheless, proton MRS will remain expensive and there is the need to sedate most patients affected with mental retardation.

Tandem mass spectrometry is a new technique by which guanidinoacetic acid and creatine can be determined [8,9,10]. Eventhough the methods are still under optimization, tandem MS already comprises a promising alternative to the previous reported methods (e.g. SID GC-MS, HPLC), which are usually more time-consuming. Furthermore, tandem mass spectrometry is applied for neonatal screening, with the potential for simultaneous multidisease screening using a single analytical technique. Screening for CCDS is not yet included in the neonatal screening programs. Nevertheless, the relatively long pre-symptomatic phase of the disease, its high frequency, the availability of treatment options and simple diagnostic tools make these disorders suitable for inclusion in the neonatal screening programs. However, X-linked SLC6A8 deficiency does not present with altered creatine and guanidinoacetic acid concentrations in blood, this defect will not be found by analysis of bloodspots.

Currently, molecular analysis is performed, most of the time by direct DNA sequencing. The field of microarrays allows the analysis of multiple genes or even full genomes in a single assay, opening a new world in both research and clinical applications. The development of sequencing chips (i.e. resequencing arrays) discloses new perspectives also in the screening for CCDS when the sensitivity of these arrays improves. Prenatal molecular diagnosis is also available in families in which an affected

child with CCDS has been diagnosed [11,5]. Conventional prenatal diagnostic procedures such as chorionic villus sampling or amniocentesis (needed for molecular or biochemical diagnosis), impart a risk of fetal loss. Detection of fetal DNA/RNA in maternal plasma is a useful tool for detecting and monitoring certain fetal diseases, opening new opportunities in non-invasive prenatal diagnosis [12]. With the future developments in this area, new options of prenatal diagnosis may also become available for CCDS.

Treatment of CCDS is one of the big challenges, in particular treatment of SLC6A8. In patients with biosynthesis defects impressive clinical improvement has been observed upon creatine supplementation (combined with ornithine supplementation and arginine restriction for GAMT deficiency), with almost complete restoration of creatine in brain [13,14]. This proves that cerebral creatine restoration is essential. However, complete reversal from the symptoms may not be achieved if not treated early in life. Treatment *in utero* may be a possibility to achieve complete restoration and to avoid clinical symptoms.

Elucidation of the creatine biosynthesis pathway in the brain as well as the clarification of the function of creatine in brain may increase the success rate of treatment. We showed that creatine is released in an action-potential dependent (exocytotic) manner. However, further studies are needed to investigate from which type(s) of central neurons the release occurs. In addition, creatine acts on $GABA_A$ receptors as a partial agonist [15]. Could creatine also act on a still unknown creatine receptor? Answering these questions will help to clarify the role of creatine in brain providing valuable information on the pathophysiology of these important syndromes.

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NEDERLANDSE SAMENVATTING

Patiënten met cerebrale creatine deficiëntie syndromen (CCDS) kunnen worden gediagnosticeerd op basis van de concentraties creatine and guanidinoazijnzuur in lichaamsvloeistoffen. In **hoofdstuk 2** wordt een stabiele isotoop dilutie gas chromatografie massa spectrometrie methode beschreven voor het gelijktijdig kwantificeren van creatine en guanidinoazijnzuur in plasma, urine and CSF. Deze methode is gebruikt voor het bepalen van referentiewaarden voor de twee metabolieten. De referentiewaarden bleken leeftijdsafhankelijk te zijn, waarbij de concentraties afnemen met de leeftijd.

Hoofdstuk 3 beschrijft overexpressie experimenten in GAMT-deficiënte fibroblasten. We hebben aangetoond dat GAMT activiteit in fibroblasten van GAMT patiënten wordt hersteld als deze cellen worden getransfecteerd met een vector met het wild type *GAMT*-gen. Omdat fibroblasten niet zo snel groeien en moeilijk te transfecteren zijn, hebben we ook experimenten met HeLa cellen uitgevoerd. De beschreven technieken kunnen worden gebruikt voor het bestuderen van DNA varianten waarvan niet duidelijk is of zij leiden tot een verminderde enzymactiviteit.

In **hoofdstuk 4** hebben we de dragerschapfrequentie van een frequente mutatie in het *GAMT* gen (W20S) onderzocht. Met de techniek die in hoofdstuk 3 beschreven staat hebben we de aard van deze mutatie onderzocht en vastgesteld dat de W20S variant een pathogene mutatie is.

In **hoofdstuk 5** hebben we de prevalentie van de X-gebonden overervende ziekte SLC6A8 deficiëntie bestudeerd. In een groep van 288 patiënten met mentale retardatie die waarschijnlijk via het X-chromosoom is overgeërfd vonden we zes pathogene mutaties. Deze bevinding suggereert dat X-gebonden SLC6A8 deficiëntie in deze groep een prevalentie van ca. 2.1% heeft.

Creatine speelt een belangrijke rol in het metabolisme van de spier. Patiënten met een CCDS hebben echter geen of weinig spierproblemen, maar voornamelijk cerebrale problemen. Dit maakt duidelijk dat creatine ook een essentiële rol speelt in het functioneren van de hersenen. Met experimenten in rattenhersenen, beschreven in **hoofdstuk 6**, hebben we aangetoond dat creatine wordt gesynthetiseerd en opgenomen

door neuronen. Ook wordt creatine vrijgemaakt in een actie-potentiaal afhankelijk exocytotisch proces, hetgeen suggereert dat creatine een neuromodulator is.

De huidige kennis over creatine metabolisme en een beschrijving van de CCDS zijn samengevat in **hoofdstuk 7**.

Hoofdstuk 8 is een korte samenvatting van dit proefschrift. Ook worden de bevindingen van het gehele onderzoek bediscussieerd en wordt een blik op de toekomst geworpen.

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CURRICULUM VITAE

Lígia Susana Vieira de Almeida was born on the 13th October 1976 in Porto, Portugal. In 1994 she started a masters in Biology (scientific area) at the Science Faculty of Porto University, which was concluded in 1999. In that period, in 1997, the course was attended at Uppsala University as part of the ERASMUS program and in 1998 the final research project was performed also at Uppsala University at the Department of Physiological Botany. From 1999 to 2000 she worked at the Institute for Cell and Molecular Biology (IBMC, Porto) with a research schoolarship. In 2000 she started in the field of the human genetics diseases, more specifically metabolic diseases at the Instituto de Genética Médica Dr Jacinto de Magalhães (IGMJM, Porto), Unidade de Biologia Clínica, where she stayed until the begining of the PhD research in 2003. The research for the PhD thesis was developed at the Metabolic Unit, Dept of Clinical Chemistry, VU University Medical Center. At present, she attends the education program, with specialization in Genetics, from the Portuguese Ministry of Health.

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